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Role of Sec16p in the Formation of COPII-Coated ER to Golgi Transport Vesicles in *Saccharomyces cerevisiae*

by

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Abstract

COPII coated transport vesicles mediate the movement of secretory proteins from the endoplasmic reticulum to the Golgi apparatus in *Saccharomyces cerevisiae*. Genetic and biochemical studies have identified seven genes that are required for the formation of COPII vesicles. *SEC12* codes for an integral ER membrane protein that regulates the initiation of vesicle budding. Five of the genes - *SEC13*, *SEC31*, *SEC23*, *SEC24*, and *SAR1* - encode the cytosolic COPII proteins that form a coat around the budded vesicle and can replace cytosol in a reaction that reconstitutes vesicle formation using ER microsomes in vitro. The role of the seventh gene, *SEC16*, in vesicle formation is less well understood. The initial, molecular characterization of *SEC16* forms the basis of this doctoral thesis.

Genetic and molecular experiments reveal that *SEC16* is essential for growth and required for vesicle budding in vivo. *SEC16* codes for an insoluble, 240 kD peripheral ER membrane protein that is tightly associated with the membrane and is incorporated into the coat of COPII vesicles that bud from the ER. In vitro binding studies demonstrate that the COPII coat proteins, Sec23p and Sec24p, bind directly to Sec16p. Sec31p has also been shown to bind to Sec16p in vitro, and this binding interaction is required in vivo. In addition to binding to the COPII proteins, Sec16p can oligomerize. Self-association occurs through the domain of Sec16p that contains each of the *sec16* temperature-sensitive mutations, indicating that oligomerization of Sec16p may be required for vesicle budding. These data suggest that Sec16p may oligomerize on the ER membrane and serve as a membrane receptor for the recruitment of COPII proteins to the vesicle bud site.

Studies using purified proteins show that the cytosolic domain of Sec12p competes with Sec23p for binding to the COOH-terminus of Sec16p. Sec12p and Sec23p function as the guanine nucleotide exchange factor and GTPase activating protein, respectively, for Sar1p, the small GTP-binding protein that regulates vesicle formation. These data point to a potential regulatory role for Sec16p in vesicle budding from the ER. In conclusion, Sec16p shows either genetic or biochemical interactions with each of the other six proteins required for COPII vesicle formation and appears to be functionally positioned at the center of the COPII vesicle assemblage.

Thesis Supervisor: Chris A. Kaiser, Associate Professor of Biology

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Chapter I

Mechanisms of Coated Vesicle Formation

Overview

Proteins targeted for secretion from eukaryotic cells travel through a series of membrane enclosed compartments en route to the cell surface. The movement of protein cargo between the different organelles of the secretory pathway is mediated by transport vesicles that bud from the donor compartment and fuse with the acceptor compartment (Palade, 1975). In the best studied examples of protein transport between organelles, vesicle formation is accompanied by the recruitment of cytosolic proteins to the membrane. These soluble proteins form a coat around the budding vesicle that is believed to play a role in both the physical formation of the vesicle and the packaging of protein cargo (Schekman and Orci, 1996). Three classes of vesicles - clathrin coated vesicles, coatomer coated vesicles and COPII coated vesicles - have been purified and the components of the vesicle coats have been characterized. Clathrin coated vesicles participate in receptor mediated endocytosis and transport of proteins from the trans-Golgi to the lysosome (Schmid, 1997). Coatomer coated vesicles function in retrograde transport of proteins from the Golgi to the endoplasmic reticulum (Cosson and Letourneur, 1997). A third class of vesicle, COPII coated vesicles, mediate transport of proteins from the ER to the Golgi (Kaiser *et al.*, 1997).

Secretory proteins are co-translationally translocated into the endoplasmic reticulum where the proteins fold and receive carbohydrate modifications (Kaiser *et al.*, 1997). Proteins destined for later compartments of the secretory pathway are packaged into vesicles for transport to the Golgi apparatus. Understanding how these COPII coated vesicles form is the focus of the experiments described in this thesis. The paradigms of clathrin coated and coatomer coated vesicles serve as a valuable foundation for the models describing COPII vesicle formation. In order to better understand the origins of

these models, the literature regarding the formation of clathrin and coatomer coated vesicles is reviewed briefly and is followed by a more detailed discussion of the components required for vesicle budding at the ER.

Clathrin Coated Vesicles

Clathrin coated vesicles mediate the endocytosis of plasma membrane receptors as well as the transport of proteins from the trans-Golgi to the lysosome. The protein coat of these vesicles consists primarily of two heteromeric complexes, clathrin and adaptor (AP) proteins (Schmid, 1997). Clathrin consists of three heavy chain and two light chain molecules. Together these proteins form a three legged structure called a triskelion. Triskelions assemble into a polygonal lattice that conforms to the emerging bud and eventually surrounds the vesicle (Schmid, 1997). The tetrameric AP complexes function to package cargo into vesicles by binding to both the cytosolic domains of integral membrane proteins and to clathrin. Two distinct, but homologous, AP complexes exist. The AP1 complex recognizes proteins such as the mannose-6-phosphate receptor for transport from the trans-Golgi compartment to the lysosome (Glickman *et al.*, 1989). The AP2 complex binds to a conserved tyrosine-based motif in plasma membrane receptors and acts in endocytosis (Sorkin and Carpenter, 1993).

The development of in vitro assays that dissect the process of vesicle budding into distinct steps has facilitated the description of the events that lead to clathrin coated vesicle formation on the plasma membrane. Formation of clathrin coated vesicles begins with the recruitment of AP2 complexes to the membrane (Fig. 1, step 1). Targeting of AP2 to the plasma membrane is not simply due to interactions with receptors since high concentrations of receptors exist in other organelles such as the endosome (Robinson, 1994). Instead,

docking proteins for AP2 may exist that initially recruit AP2 to the plasma membrane prior to binding to receptor domains (Fig. 1). Recruitment of AP2 complexes from cytosol in vitro requires ATP and is inhibited by GTPγS, which suggests the possible involvement of a GTPase (Seaman *et al.*, 1993).

Binding of AP1 complexes to trans-Golgi membranes has also been reconstituted in vitro. Recruitment of AP1 is blocked by treatment with brefeldin A and stimulated by GTPγS (Robinson and Kreis, 1992). These experiments suggested that the brefeldin A-sensitive small GTP-binding protein ARF may be involved in AP1 membrane binding. Further studies showed that depletion of ARF from cytosol prevents binding of AP1, but that addition of recombinant ARF restores binding (Traub *et al.*, 1993; Stamnes and Rothman, 1993). Although no small GTP-binding protein has been shown to be required in AP2 recruitment, ARF appears to be required for binding of AP1 to high affinity binding sites on the trans-Golgi that include the mannose-6-phosphate receptor (Le Borgne *et al.*, 1996).

AP complexes bound to membrane receptors nucleate the assembly of clathrin triskelions into planar lattices that gradually transform into coated pits or buds (Fig. 1, steps 2-4; Schmid, 1997). Clathrin and AP complexes contact each other directly and can form coats in vitro (Shih *et al.*, 1995; Schmid, 1997). Spontaneous assembly of these components in the cytosol appears to be inhibited by phosphorylation. AP2 complexes in the cytosol are phosphorylated and are unable to bind to clathrin cages, whereas AP2 complexes bound to the membrane are not phosphorylated (Wilde and Brodsky, 1996). These data suggest that dephosphorylation of AP2 after recruitment to the membrane may permit binding to clathrin and facilitate coat formation.

Addition of clathrin and AP2 complexes to membranes results in the formation of clathrin coated buds in vitro. Experiments using this assay

demonstrate that additional cytosolic proteins, ATP, and GTP hydrolysis are required for subsequent steps, such as constriction of the bud neck and eventual membrane fission (Schmid and Smythe, 1991). Constricted coated pits are buds in which small molecules, but not macromolecules such as antibodies, can access receptor bound ligands.

One protein directly involved in the formation of constricted coated pits and the completed vesicle is the 100 kD GTPase dynamin. Mutational studies of dynamin in both mammalian cells and Drosophila indicate that inhibition of dynamin function in vivo allows formation of invaginated clathrin coated pits, but blocks the formation of constricted buds (Kosaka and Ikeda, 1983; Damke *et al.*, 1994). Dynamin binds to AP2 complexes in vitro and localizes to the necks of constricted clathrin coated vesicles in nerve terminals treated with GTPγS, a non-hydrolyzable analog of GTP (Wang *et al.*, 1995; Takei *et al.*, 1995). Furthermore, dynamin can self-associate into helical stacks of rings (Hinshaw and Schmid, 1995). From these data as well as additional experiments, the following model for clathrin coated vesicle formation has emerged (Fig. 1, steps 4-6). Dynamin in the GDP-bound state is recruited to the clathrin lattice composed of clathrin and AP proteins. Exchange of GTP for GDP causes dynamin to redistribute to the neck of the budding vesicle and promotes the assembly of helical ring structures that constrict the bud neck. GTP hydrolysis by dynamin may result in a conformational change that tightens the collar-like structure and eventually leads to vesicle scission (Schmid, 1997). Formation of clathrin coated vesicles at the trans-Golgi membrane is thought to occur by a similar mechanism although a dynamin-like molecule for this process has not been identified in mammalian cells.

Coatomer Coated Vesicles

Work in mammalian cells identified a new class of Golgi-derived non-clathrin coated vesicles, called coatomer coated vesicles. Coatomer coated vesicles bud from the Golgi apparatus after incubation of Golgi membranes with cytosol and ATP in an assay that reconstitutes intra-Golgi transport in vitro (Balch *et al.*, 1984; Orci *et al.*, 1986). Addition of GTPγS to this assay blocks intra-Golgi transport and results in the formation of vesicles with a stable protein coat (Malhotra *et al.*, 1989). Purification of these vesicles on a large scale allowed the characterization of the protein coat which contains two major components: the small GTP-binding protein ARF and a seven subunit, cytosolic protein complex, called coatomer (Waters *et al.* 1991; Serafini *et al.*, 1991). Biochemical analysis of the vesicles revealed that ARF is a stoichiometric component of the synthesized vesicles (Serafini *et al.*, 1991). In vitro experiments demonstrated that ARF and GTP are sufficient for the recruitment of coatomer and the formation of vesicles from Golgi membranes (Orci *et al.*, 1993). These data have led to a model for the formation of coatomer coated vesicles from Golgi membranes (Rothman, 1994). Myristylated ARF-GDP is recruited to the Golgi membrane through an interaction with an ARF receptor that is likely a guanine nucleotide exchange factor for ARF. The exchange factor catalyzes binding to GTP which results in activation of ARF and binding to the membrane by means of the attached myristic acid moiety. Activated ARF would then recruit coatomer leading to the formation of vesicles with a coat composed of ARF and coatomer. A candidate for the ARF receptor has been recently identified. The human protein ARNO enhances ARF nucleotide exchange in vitro, but it remains to be shown that ARNO localizes to the Golgi (Chardin *et al.*, 1996).

Recent experiments suggest that ARF may not be an obligatory

component of coatomer coated vesicles, but may instead act to recruit coatmer by stimulating phospholipase D (PLD) activity. ARF can activate Golgi-localized PLD which leads to the production of phosphatidic acid and choline from phosphatidylcholine (Brown *et al.* 1993; Cockcroft *et al.*, 1994). Coatomer has been shown to bind to either Golgi membranes treated with bacterial PLD or to artificial phospholipid vesicles as a function of the content of phosphatidic acid in the absence of added ARF (Ktistakis *et al.*, 1996). One hypothesis is that ARF activates the localized production of phosphatidic acid which results in the binding of coatomer and vesicle budding. Consistent with this, Golgi membranes isolated from cells expressing high levels of PLD activity do not require ARF for the formation of coatomer coated vesicles in vitro and ARF is not a component of these vesicles (Ktistakis *et al.*, 1996). Likewise, reisolated Golgi membranes that have been preincubated with ARF do not require ARF for the formation of vesicles and again ARF is not found as a stoichiometric component of the vesicle coat (Ktistakis *et al.*, 1996). These data have refocused attention on the role of lipids in vesicle formation.

A homologous coatomer complex exists in *Saccharomyces cerevisiae* (Hosobuchi *et al.*, 1992). Genetic studies involving the genes that encode these coatomer subunits have suggested a functional role for coatomer coated vesicles in retrograde transport of proteins from the Golgi to the ER (Cosson and Letourneur, 1997). ER membrane proteins containing a -KKXX signal on their cytoplasmic tails are believed to be localized to the ER by continuous retrieval from post-ER compartments (Townsley and Pelham, 1994). Mutations in the coatomer subunits cause defects in the retention of resident ER proteins containing the -KKXX retrieval signal (Letourneur *et al.*, 1994). In addition, coatomer subunits bind to fusion proteins containing the -KKXX retention signal in vitro (Cosson and Letourneur, 1994). By analogy to the mechanism of cargo

sorting in clathrin coated vesicles, these data suggest that coatomer binds to the cytoplasmic tails of -KKXX proteins and helps to package the proteins into coatomer coated vesicles that deliver the proteins back to the ER. These genetic data in yeast have raised questions regarding the role of coatomer in intra-Golgi transport (Pelham, 1994). In addition, experiments in both yeast and mammalian cells suggest that coatomer coated vesicles may play a role in ER to Golgi transport (Pepperkok *et al.*, 1993; Bednarek *et al.*, 1995; Aridor *et al.*, 1995) However, recent experiments indicate that coatomer may function in both anterograde intra-Golgi and retrograde transport and thus participate in multiple vesicle trafficking steps (Orci *et al.*, 1997).

COPII Coated Vesicles

Experiments in the yeast *Saccharomyces cerevisiae* and mammalian cells revealed a third class of coated vesicle, the COPII vesicle, that mediates transport of proteins from the endoplasmic reticulum to the Golgi apparatus. The majority of what is known about this system comes from work in yeast, but the discovery of a homologous mechanism in mammalian cells demonstrates the universality of this step in protein transport to the Golgi.

The use of both genetic and biochemical approaches facilitated the description of the mechanism of ER to Golgi transport vesicle formation. A screen for conditional yeast mutants defective for secretion and cell surface growth identified 23 complementation groups (Novick and Schekman, 1980). A subset of these *sec* mutants showed a defect in the transport of a secretory marker protein to the Golgi apparatus (Novick *et al.*, 1981). Detailed morphological analysis revealed that the ER to Golgi transport mutants can be divided into two classes: genes necessary for the formation of vesicles and genes required for the fusion of these vesicles with the Golgi (Fig. 2) (Kaiser

and Schekman, 1990). Mutations in the genes required for vesicle fusion (*sec17*, *sec18*, and *sec22*) cause an accumulation of 50 nm vesicles at the non-permissive temperature. Mutants defective for vesicle formation (*sec12*, *sec13*, *sec16*, and *sec23*) accumulate excess ER membrane due to an inability to bud vesicles. Epistasis tests between genes from the two classes of mutants confirm that the vesicle formation genes act at an earlier step than the fusion genes (Kaiser and Schekman, 1990). Furthermore, mutants in the same class exhibit synthetic lethal interactions suggesting that the mutations affect a single function (Fig. 2).

The reconstitution of ER to Golgi transport in vitro permitted the biochemical dissection of the requirements for the formation of ER transport vesicles. An assay that measures the transport of a secretory protein, α-factor, from the ER to the Golgi was developed using gently lysed yeast spheroplasts (Baker *et al.*, 1988; Ruohola *et al.*, 1988). Briefly, in vitro synthesized radiolabelled α-factor is post-translationally translocated into the ER lumen where it is core glycosylated. Incubation of semi-intact yeast with cytosol, ATP and GTP promotes transport to the Golgi which results in the addition of Golgi-specific outer chain carbohydrates to α-factor. The efficiency of transport is quantitated as the fraction of core glycosylated α-factor that receives outer chain modifications. Typically, 25-50% of the core glycosylated α-factor is transported to the Golgi (Kaiser *et al.*, 1997).

A simplified version of this assay using semi-intact yeast cells as the donor membranes allows examination of the vesicle budding step in the absence of vesicle fusion. Vesicle budding is measured as the appearance of core glycosylated α-factor in slowly sedimenting vesicles that have been separated from the more rapidly sedimenting donor membranes by differential centrifugation (Groesch *et al.*, 1990; Rexach and Schekman, 1991). Vesicles

produced in this assay fuse with semi-purified Golgi acceptor membranes demonstrating that the vesicles produced are functional intermediates of ER to Golgi transport (Rexach and Schekman, 1991; Ruohola *et al.*, 1991). Studies of membranes and cytosol prepared from mutant strains demonstrated that the products of the *sec12* and *sec23* genes are required for efficient vesicle formation in addition to cytosol, ATP and GTP (Rexach and Schekman, 1991). The convergence of in vitro and in vivo results established the in vitro assay as a useful model of ER to Golgi transport.

Wild-type yeast are not a rich source of ER transport vesicles. Consequently, studies have relied on the analysis of vesicles synthesized in vitro using either ER microsomes or perforated yeast defective in vesicle fusion with the Golgi (Barlowe *et al.*, 1994; Rexach *et al.*, 1994). Large scale purification of the vesicles synthesized from ER microsomes yields a homogeneous population of 60-65 nm vesicles with an electron-dense coat (Barlowe *et al.*, 1994). These vesicles are functional intermediates in the secretory pathway as purified vesicles can fuse and deliver α-factor to the Golgi (Barlowe *et al.*, 1994; Rexach *et al.*, 1994). Furthermore, in vitro vesicle budding reconstitutes the specificity of cargo sorting seen in vivo. Vesicles contain proteins required for targeting and fusion with the Golgi such as Sec22p and Bet1p, but do not contain resident ER proteins such as Kar2p/BiP (Salama *et al.*, 1993). In addition to targeting molecules, vesicles that have lost the protein coat also contain twelve abundant integral membrane proteins, termed ERV (ER vesicle protein) (Rexach *et al.*, 1994).

Fractionation of the cytosol requirement for the in vitro budding assay demonstrated that five soluble proteins - Sec23p/Sec24p, Sec13p/Sec31p and the small GTP-binding protein Sar1p - can substitute for cytosol in a reaction using urea washed ER microsomes or semi-intact cells and GMP-PNP, a non-

hydrolyzable analog of GTP (Salama *et al.*, 1993; Barlowe *et al.*, 1994). Analysis of the vesicles produced in this reaction using immuno-electron microscopy revealed that the electron dense coat consists of the five soluble proteins required for vesicle synthesis in vitro. Vesicles formed in this reaction are morphologically and molecularly distinct from both clathrin coated and coatomer (COPI) coated vesicles and are thus called COPII vesicles (Barlowe *et al.*, 1994). The proteins that constitute the coat are referred to as COPII proteins.

The in vitro budding assay has been useful in identifying the soluble proteins required to form vesicles from urea washed membranes. However, uncovering the essential components of the donor membranes has been more difficult. Three other proteins, Sec16p, Sec12p and Sed4p, are involved in vesicle formation as determined by genetic methods. *SEC16* , isolated in the original screen for secretion defective mutants, is required for vesicle formation in vivo (Novick and Schekman, 1980; Kaiser and Schekman, 1990). *SEC12* and *SED4* are integral membrane proteins that regulate vesicle formation, but are not components of the completed COPII vesicle (Barlowe *et al.*, 1994; Gimeno *et al.*, 1995). Proteins that are present in a sufficient quantity as components of the urea washed donor membranes will not appear to be required in the in vitro budding assay. Thus, additional integral membrane or peripheral membrane proteins may be required for the formation of vesicles.

Sec23p and Sec24p: Cytosolic COPII vesicle coat proteins

SEC23 was initially identified as a mutant defective in secretion and was later shown to be specifically required for vesicle budding from the ER (Novick and Schekman, 1980; Kaiser and Schekman, 1990). Molecular cloning of *SEC23* revealed that the gene codes for an 84 kD soluble protein that is

present in the cytosol and on the ER membrane (Hicke and Schekman, 1989). Membranes and cytosol prepared from a *sec23* mutant strain display a temperature-dependent defect in ER to Golgi transport in vitro (Baker *et al.*, 1988). Addition of wild-type cytosol to the reaction corrects the defect, providing an assay for the purification of Sec23p activity. Purified Sec23p activity fractionates as a 300-400 kD complex by gel filtration chromatography and consists of Sec23p and a 105 kD polypeptide, Sec24p (Hicke *et al.*, 1992). Sec24p, like Sec23p, is essential for growth and is required for vesicle formation in vivo and in vitro (Hicke *et al.*, 1992; Kaiser *et al.*, 1997). Together Sec23p/Sec24p constitute a major component of the COPII vesicle coat and are two of five cytosolic proteins required for vesicle budding in vitro (Barlowe *et al.*, 1994). The stoichiometry of the complex is still not known.

In wild-type yeast cells, Sec23p exists in a complex with Sec24p, but overexpression of *SEC23* results in the accumulation of monomeric Sec23p in the cytosol. Addition of excess monomeric Sec23p to the in vitro budding reaction inhibits vesicle formation. The addition of increased amounts of the small GTP-binding protein Sar1p partially relieves this inhibition and indicates a functional interaction between the two proteins (Yoshihisa *et al.*, 1993). Indeed, Sec23p functions as a GTPase activating protein (GAP) specifically for Sar1p and increases the GTPase activity of Sar1p by 10-15 fold (Yoshihisa *et al.*, 1993). The effect of Sec23p on Sar1p GTPase activity is due to an increase in the rate of hydrolysis and not an increase in the rate of exchange of GDP for GTP (Yoshihisa *et al.*, 1993). The presence of Sec24p has no effect on Sec23p GAP activity, and the exact function of Sec24p remains uncertain.

Sec13p and Sec31p: Cytosolic COPII vesicle coat proteins

Genetic and morphological analysis identified *SEC13* as a gene

required for vesicle formation in vivo (Novick and Schekman, 1980; Kaiser and Schekman, 1990). *SEC13* exhibits genetic interactions with *SEC12*, *SEC16*, and *SEC23* indicating that it likely functions with these other gene products in vesicle budding. Molecular analysis of *SEC13* demonstrates that the gene codes for a hydrophilic protein of 33 kD that is largely cytosolic (Pryer *et al.*, 1993). Membrane-associated Sec13p can be removed by treatment with 2.5 M urea. Cytosol depleted of Sec13p is defective for vesicle budding in vitro using urea washed ER microsomes (Pryer *et al.*, 1993). Addition of cytosol to this reaction restores budding and provides a means for the purification of the Sec13p activity. Sec13p exists in a 700 kD complex formed by two polypeptides, Sec13p and Sec31p (Salama *et al.*, 1993). Sec31p is a 150 kD protein that is essential for growth and required for ER to Golgi transport both in vitro and in vivo (Wuestehube *et al.*, 1996; Salama *et al.*, 1997). Although the Sec13p/Sec31p complex fractionates as a 700 kD complex by gel filtration chromatography, glycerol gradient analysis suggests that Sec13p/Sec31p is a 166 kD heterodimer (Salama *et al.*, 1997). Sec13p/Sec31p, Sec23p/Sec24p, and Sar1p are the minimal components for vesicle budding from urea washed membranes (Salama *et al.*, 1993). In addition, Sec13p/Sec31p complex is a major component of the COPII vesicle coat (Barlowe *et al.*, 1994).

Interestingly, both Sec13p and Sec31p have multiple repeats of the conserved WD motif found in β subunits of heterotrimeric G proteins (Pryer *et al.*, 1993; Salama *et al.*, 1997). Proteins containing WD repeats have been found to function in a wide variety of cellular processes (Neer *et al.*, 1996). Crystallographic analysis of this protein domain demonstrates that the repeats fold into a β-propeller structure that is hypothesized to mediate protein-protein interactions (Sondek *et al.*, 1996). Consistent with this model, binding of Sec31p to Sec13p is mediated by the domain in Sec31p containing WD

repeats (Shaywitz *et al.*, 1997).

Sec13p and Sec31p are clearly essential for the formation of ER transport vesicles, but the exact role of the proteins in this process is unclear. Detailed analysis of Sec31p binding interactions reveals that Sec31p binds to Sec13p, Sec23p, Sec24p, and Sec16p, all components of the COPII coat (Shaywitz *et al.*, 1997). Studies using a deletion mutant of Sec31p suggest that binding of Sec31p to Sec16p is required for ER to Golgi transport (Shaywitz *et al.*, 1997). The discovery of binding interactions between the two major COPII subunit complexes, Sec23p/Sec24p and Sec13p/Sec31p, is consistent with a model in which the COPII coat assembles by the polymerization of its subunits.

Despite the apparent essential requirement of Sec13p for vesicle formation, *SEC13* may not always be required for ER to Golgi transport. Loss of function mutations in three genes - *BST1*, *EMP24/BST2*, and *ERV25/BST3* - can bypass the requirement for *SEC13* in vivo (Elrod-Erickson and Kaiser, 1996; Belden and Barlowe, 1996). Mutations in these genes have only minor effects on protein transport to the Golgi. Transport of a subset of secretory proteins to the Golgi is slowed in these mutants, while ER resident proteins show an increased rate of transit to the Golgi (Schimmöller *et al.*, 1995; Elrod-Erickson and Kaiser, 1996). These data indicate that mutations in *BST1*, *BST2*, and *BST3* cause a decrease in the fidelity of cargo sorting at the ER and that the products of these three genes may function as a checkpoint that monitors cargo sorting and prevents aberrant vesicle formation (Elrod-Erickson and Kaiser, 1996). Emp24p/Bst2p and Erv25p/Bst3p are both abundant integral membrane components of COPII vesicles (Schimmöller *et al.*, 1995; Belden and Barlowe, 1996). An alternative proposal is that these proteins function as cargo sorting receptors for a specific subset of secretory proteins (Schimmöller *et al.*, 1995; Belden and Barlowe, 1996).

In addition to a role in vesicle formation, Sec13p may function in other processes that do not involve the COPII proteins. Sec13p and a homologous protein Seh1p were isolated as components of a nuclear pore protein complex (Siniossoglou *et al.*, 1996). Sec31p is not present in this complex. Genetic analysis suggests a functional role for *SEH1* in nuclear transport, but not for *SEC13*. *SEH1* appears to function specifically in nuclear transport since mutations in *SEH1* did not affect ER to Golgi transport (Siniossoglou *et al.*, 1996). It remains to be seen if the association of Sec13p with the nuclear pore complex has any functional significance.

Sec13p has also been shown to function at a late step in the secretory pathway that involves the transport of amino acid permeases from the Golgi to the plasma membrane (Roberg *et al.*, 1997). Mutations in *SEC13* cause Gap1p, an amino acid permease, to be transported to the vacuole instead of the plasma membrane. Mutations in other COPII genes, such as *SEC12*, *SEC16*, *SEC23*, and *SEC31*, have no effect on Gap1p transport from the Golgi (Roberg *et al.*, 1997). These data raise the possibility that Sec13p may be a component of another vesicle coat that functions at a later step in the secretory pathway.

Sar1p: A small GTP-binding protein

SAR1 was isolated as a multi-copy suppressor of a temperature-sensitive *sec12* mutant (Nakano and Muramatsu, 1989). *SAR1* encodes an essential small GTP-binding protein that is most closely related to the GTP-binding protein ARF that functions in the formation of coatomer coated vesicles (Oka *et al.*, 1991; Barlowe *et al.*, 1993). Indirect immunofluorescence experiments show that Sar1p localizes to the nuclear periphery and to spots in the cytoplasm (Nishikawa and Nakano, 1991). Sar1p is tightly associated with membranes in cell lysates and is solubilized by deoxycholate, but not by treatment with 1M

NaCl or high pH (Nishikawa and Nakano, 1991). Unlike other small GTP-binding proteins such as Ras and ARF, Sar1p appears not to contain a lipid modification that assists in binding to membranes. Sar1p lacks the consensus amino acid sequences for these modifications and bacterially expressed recombinant Sar1p is fully functional in vitro (Oka *et al.*, 1991; Barlowe *et al.*, 1994). The ability to establish a Sar1p-dependent in vitro budding assay facilitated the purification of Sar1p from yeast lysates (Barlowe *et al.*, 1993). Purified Sar1p has an intrinsic rate of GTP hydrolysis (0.0011 min^{-1}) and guanine nucleotide off-rate (0.07 min^{-1}) similar to other GTPases (Barlowe *et al.*, 1993).

The activity of small GTPases is often controlled by regulatory proteins in vivo (Bourne *et al.*, 1991). *SAR1* displays genetic interactions with two genes known to regulate its activity, *SEC12* and *SEC23*. Sec12p functions as a guanine nucleotide exchange factor for Sar1p and stimulates the dissociation of GDP (Barlowe and Schekman, 1993). Sec23p functions as a GTPase activating protein and catalyzes hydrolysis of GTP by Sar1p (Yoshihisa *et al.*, 1993). Together, these three proteins perform a cycle of GTP binding and hydrolysis. Incubation of Sar1p with Sec23p increases the rate of hydrolysis 10 fold. But, incubation of Sar1p with both Sec12p and Sec23p allows the GTPase to cycle and increases the hydrolysis rate 50 fold (Barlowe and Schekman, 1993).

Sar1p and GTP are required for vesicle budding in vitro (Oka and Nakano, 1994; Barlowe *et al.*, 1994). However, hydrolysis of GTP is not required for vesicle budding, but it is necessary for fusion of vesicles with the Golgi (Oka and Nakano, 1994; Barlowe *et al.*, 1994). Vesicles produced in vitro using GMP-PNP have a stable coat that is composed of the COPII proteins, Sar1p, Sec23p/Sec24p, and Sec13p/Sec31p. The presence of this coat

prevents fusion with the Golgi. Vesicles synthesized using GTP have a coat that does not contain Sar1p and disassembles after incubation on ice (Barlowe *et al.*, 1994). These data have led to the model that Sar1p bound to GTP promotes vesicle coat assembly, whereas hydrolysis of GTP by Sar1p promotes disassembly of the vesicle coat. Coat disassembly likely occurs in a two-step process in which Sar1p is first lost from the coat followed by the dissociation of the Sec23p and Sec13p complexes (Barlowe *et al.*, 1994).

Sec12p: Regulator of Sar1p activity

SEC12 codes for an essential, 70 kD ER integral membrane protein that is required for COPII vesicle formation in vivo, but is not incorporated into vesicle in vitro (Nakano *et al.*, 1988; Kaiser and Schekman, 1990; Nishikawa and Nakano, 1993; Barlowe *et al.*, 1994). Several lines of evidence indicate that the functions of *SEC12* and *SAR1* are intimately linked. Genetic experiments demonstrate that overexpression of *SAR1* suppresses the growth defect of a *sec12* temperature-sensitive mutant (Nakano and Muramatsu, 1989). Suppression of a *sec12* defect by Sar1p is also observed in vitro. Membranes prepared from a *sec12* mutant strain display an ER to Golgi transport defect in vitro, but addition of Sar1p restores normal vesicle transport (Rexach and Schekman, 1991; Oka *et al.*, 1991). Sar1p binding to isolated membranes increases upon the overexpression of Sec12p, suggesting a physical interaction between the two proteins (d'Enfert *et al.*, 1991). Lastly, microsomes prepared from a *SEC12* overexpression strain inhibit vesicle budding when added in vitro, and this inhibition can be rescued by the addition of Sar1p (d'Enfert *et al.*, 1991).

The cytosolic domain of Sec12p (amino acids 1-354) purified from yeast functions as a guanine nucleotide exchange factor for Sar1p (Barlowe and

Schekman, 1993). Sec12p does not significantly affect the rate of GTP hydrolysis of Sar1p, but stimulates the dissociation of GDP from Sar1p by 8 fold (Barlowe and Schekman, 1993). Sec12p containing a *sec12-1* temperature-sensitive mutation showed reduced exchange activity for Sar1p which suggests that the exchange activity of Sec12p is necessary for ER to Golgi transport (Barlowe and Schekman, 1993). Sec12p may recruit Sar1p to the ER by catalyzing exchange of GDP for GTP. Proper Sec12p function requires that the protein is anchored in the ER membrane since addition of the catalytic, cytosolic domain of Sec12p inhibits vesicle budding in vitro. Once again, normal budding is restored by addition of Sar1p (Barlowe and Schekman, 1993).

Sed4p

Sed4p is a type II ER transmembrane protein that is 45% identical to Sec12p in the NH_2-terminal and transmembrane domains (Hardwick *et al.*, 1992) Similar to Sec12p, Sed4p is not incorporated into COPII vesicles in vitro (Gimeno *et al.*, 1995). Mutations in *SED4* cause defects in ER to Golgi transport, and extensive genetic interactions with *SAR1* and *SEC16* suggest a role for *SED4* in vesicle budding. In addition, the cytosolic domain of Sed4p binds to Sec16p (Gimeno *et al.*, 1995). However, genetic tests demonstrate that *SED4* and *SEC12* are not functionally interchangeable, and there is no evidence that Sed4p acts as a guanine nucleotide exchange factor for Sar1p (Gimeno *et al.*, 1995).

SEC16

Temperature-sensitive mutations in *SEC16* were isolated in the original screen for secretion mutants (Novick and Schekman, 1980). Genetic and morphological studies demonstrate that *SEC16* is required for the formation of

ER transport vesicles in vivo (Kaiser and Schekman, 1990). Genetic interactions with other genes known to be involved in vesicle budding such as *SEC12*, *SEC13*, *SEC23*, and *SAR1* are consistent with this functional assignment (Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990). A recent study shows that ER microsomes prepared from *sec16* temperature-sensitive strains are defective for the packaging of membrane proteins into vesicles as compared to α-factor in vitro (Campbell and Schekman, 1997). These data suggest that Sec16p may participate in vesicle cargo loading.

COPII Vesicle Formation in Mammalian Cells

A growing body of evidence indicates that COPII vesicle formation at the ER occurs in an identical manner in mammalian cells as in yeast. The identification of mammalian homologues of yeast *SEC13*, *SEC23*, and *SAR1* have facilitated this analysis. Each of the homologues shows a high degree of identity (> 50%) with the corresponding yeast gene. Consistent with a function in vesicle budding, each of the mammalian proteins - Sec23A, Sar1a, and Sec13R - localizes to the transitional region of the ER in mammalian cells (Paccaud *et al.*, 1996; Kuge *et al.*, 1994; Shaywitz *et al.*, 1995). Two homologues of *SAR1*, Sar1a and Sar1b, were identified using degenerate oligonucleotide primers and PCR (Kuge *et al.*, 1994). Mutant forms of Sar1p and antibody to Sar1a block transport of the marker protein VSV-G to the Golgi in vitro (Kuge *et al.*, 1994). Chimeric gene constructions between human SEC13R and yeast *SEC13* complement a *sec13* temperature-sensitive mutation demonstrating a conservation of function between the two genes. In addition, antibodies directed against human Sec13Rp inhibit ER to Golgi transport in semi-intact cells (Tang *et al.*, 1997). A human counterpart for Sec31p likely exists since human Sec13Rp binds to yeast Sec31p in the two-

hybrid assay (Shaywitz *et al.*, 1997). Two human homologues of *SEC23* were identified by screening a cDNA library with a mouse *SEC23* probe (Paccaud *et al.*, 1996). Human Sec23A is a functional homologue of *SEC23* since Sec23A can complement the temperature-sensitivity of a *sec23* mutant in vivo. Like yeast Sec23p, human Sec23Ap is in a complex of ~350 kD with a second protein of ~110 kD that is most likely human Sec24p (Paccaud *et al.*, 1996). Homologues for *SEC12* and *SEC16* remain to be isolated. The identification of these homologues now permits a detailed analysis of the mechanism of vesicle budding in mammalian cells.

A Model for the Assembly of COPII Vesicles

The biochemical and genetic data discussed above have led to the development of a model for the mechanism and regulation of COPII vesicle formation (Fig. 3). In this model, vesicle formation is initiated by the recruitment of Sar1p to the ER membrane through an interaction with the guanine nucleotide exchange factor Sec12p. Sec12p catalyzes the exchange of GDP for GTP by Sar1p and activates Sar1p for binding to the ER membrane. Activated Sar1p recruits Sec23p/Sec24p and Sec13p/Sec31p to the membrane leading to vesicle budding. Hydrolysis of GTP by Sar1p, possibly stimulated by the GTPase activating protein Sec23p, results in the dissociation of Sar1p from the vesicle membrane and the disassembly of the vesicle coat. Lastly, the COPII subunits are released into the cytosol and are available for further rounds of vesicle budding.

The mechanism by which cargo is packaged into COPII vesicles is unknown. By analogy to cargo sorting in clathrin and coatomer coated vesicles, it has been proposed that subunits of the COPII vesicle coat may contact cargo directly, but currently no such interaction has been reported (Schekman and

Orci, 1996). As is the case with the other classes of coated vesicles, we have little understanding of how the COPII vesicle pinches off and separates from the membrane. Lessons from how vesicles fuse with membranes may provide clues in the future.

The current model of COPII vesicle formation assigns a function to each of the genes known to be required for vesicle formation in vivo with the exception of *SEC16*. The focus of my dissertation research has been to understand the role that the *SEC16* gene plays in the process of COPII vesicle formation. A combination of molecular, genetic, and biochemical approaches have been used to discern the function of this essential gene. An initial characterization determined the cellular localization and physical properties of Sec16p. In vitro and two hybrid binding studies identified multiple binding partners for Sec16p, and these interactions have been analyzed in detail. Lastly, the role of Sec16p in the regulation of vesicle formation was examined. Experiments suggest that Sec16p may function both to regulate the initiation of vesicle formation and to serve as a structural component of the completed vesicle.

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Figure 1. Mechanism of clathrin coated vesicle formation in receptor mediated endocytosis. *Step 1*, AP2 recruitment; *step 2*, clathrin assembly; *step 3*, dynamin recruitment; *step 4*, coated pit invagination; *step 5*, coated pit constriction requiring the redistribution of dynamin from the lattice and its assembly at the neck; *step 6*, coated vesicle budding; *step 7*, clathrin release; *step 8*, AP2 release. Reproduced with permission from Schmid, 1997.

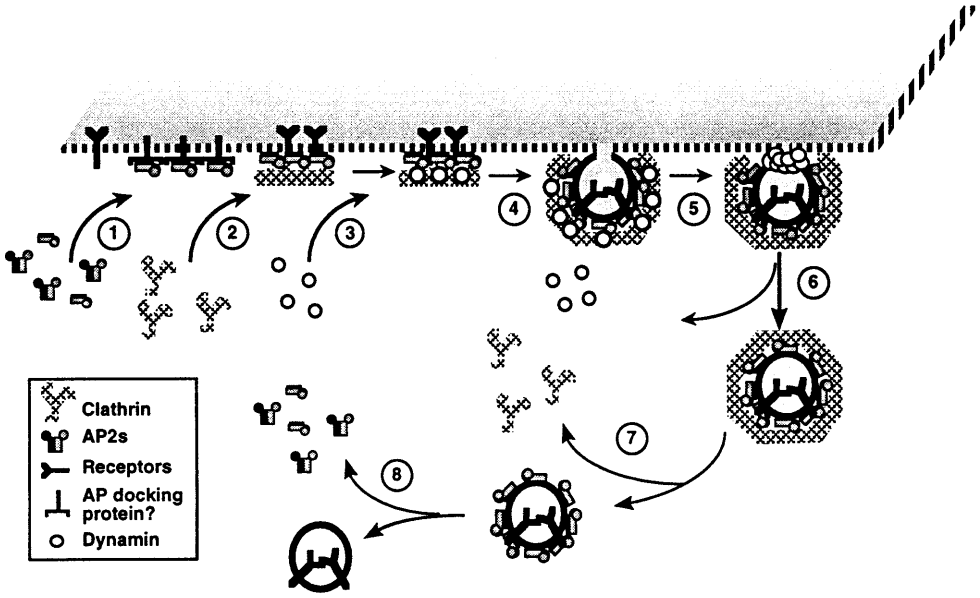

Clathrin
AP2s
Receptors
AP docking protein?
Dynamin
1
2
3
4
5
6
7
8

Figure 2. Genes required for ER to Golgi transport in *Saccharomyces cerevisiae*. Genes necessary for vesicle formation are listed on the left. Genes required for vesicle fusion are listed on the right. Adapted from Gimeno, 1996.

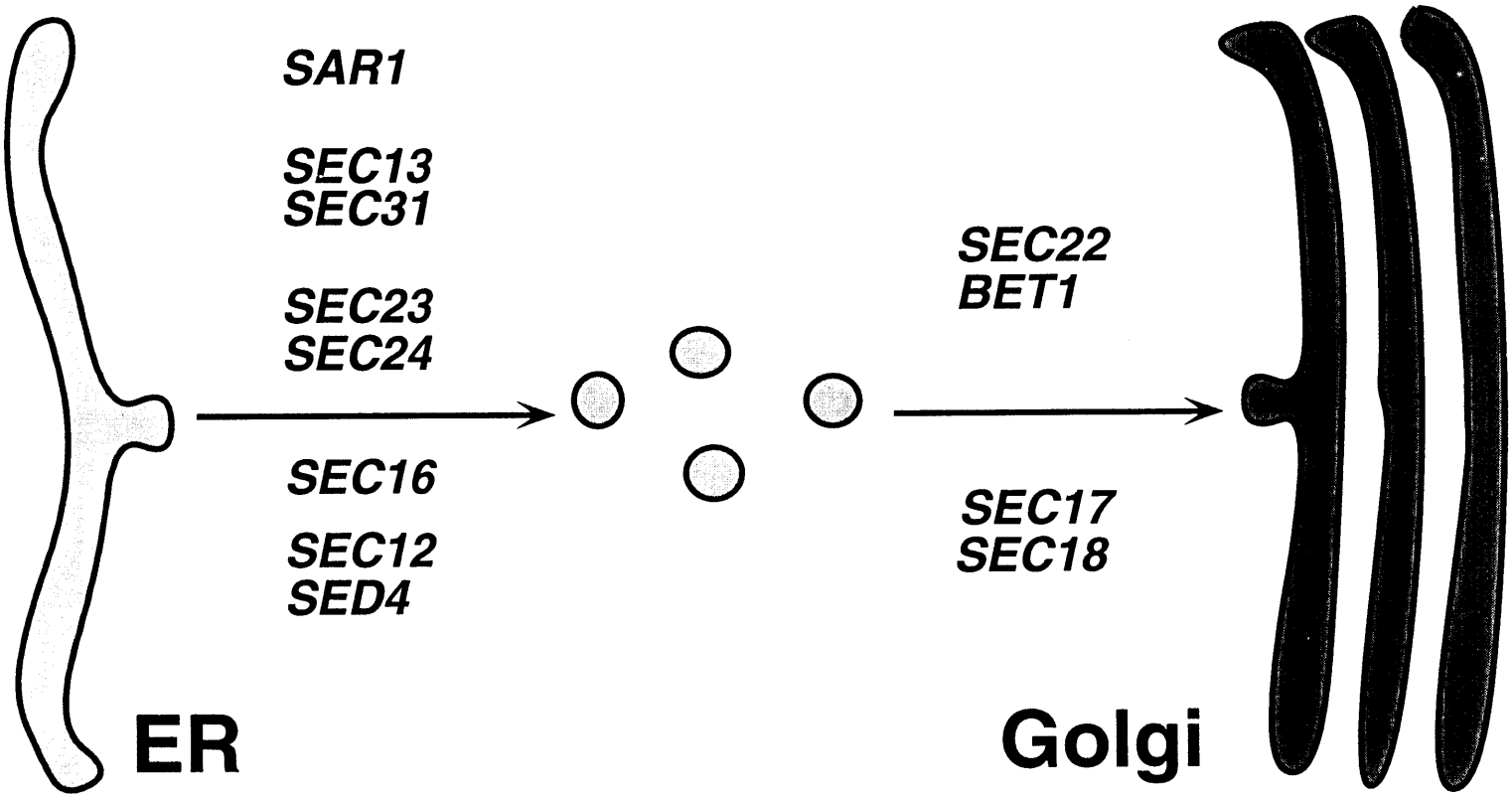
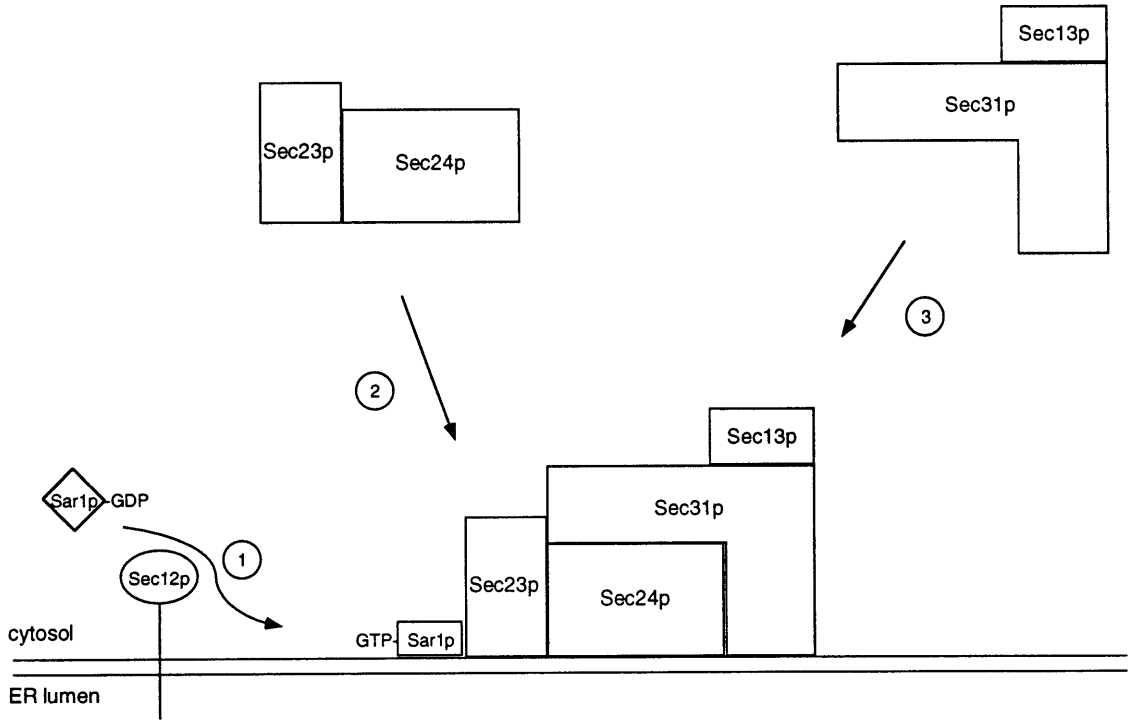

SAR1
SEC13
SEC31
SEC23
SEC24
SEC16
SEC12
SED4
SEC22
BET1
SEC17
SEC18
ER
Golgi

Figure 3. Mechanism of COPII vesicle formation at the ER. *Step 1*, recruitment of Sar1p to the ER membrane by Sec12p and activation of Sar1p by exchange of GDP for GTP; *step 2*, recruitment of Sec23p/Sec24p by Sar1p-GTP, *step 3*, binding of Sec13p/Sec31p through interactions with Sec23p/Sec24p.


Sec13p
Sec31p
Sec23p
Sec24p
3
2
Sec13p
Sec31p
Sar1p
GDP
1
Sec12p
Sec23p
Sec24p
GTP
Sar1p
cytosol
ER lumen

Chapter II

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

Preface

This chapter has been published in the Journal of Cell Biology: Espenshade, P., R.E. Gimeno, E. Holzmacher, P. Teung, and C.A. Kaiser. 1995. Yeast SEC16 gene encodes a multidomain vesicle coat protein that interacts with Sec23p. *J. Cell Biol.* 131:311-324.

Elizabeth Holzmacher and Pansy Teung cloned and sequenced the *SEC16* gene. Ruth Gimeno assisted in the testing of the two-hybrid interactions between *SEC23* and *SEC16*.

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 that are 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.

Introduction

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, approximately 20 yeast gene products have been identified that act in vesicle transport between the ER and Golgi apparatus (reviewed in Pryer *et al.*, 1992). Some of these gene products appear to be evclutionarily 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 ER and Golgi 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).

Four genes, *SEC12*, *SEC13*, *SEC16* and *SEC23,* are re ognized 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 (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 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, *SEC16* 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 CEN, *URA3* vector pRS316 (Sikorski and Hieter, 1989). The smallest complementing subclone contained a 7.2 kb *Bam*HI – *Sph*I 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 (USB, 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 *Sph*I – *Stu*I fragment containing approximately 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 *Pst*I prior to 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 performed several tests to establish that the putative initiation ATG which was preceded by in-frame stop codons was in fact the start of translation. First, a frame shift mutation was generated by a two base-pair deletion at a *Sac*II site at the codon for amino acid 103. A clone with this mutation did not complement a *sec16-2* mutation, indicating that the *Sac*II 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 about 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 *Xba*I and *Bst*XI prior to 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 *Kpn*I - *Stu*I fragment from the 3' non-coding sequence and a *Bam*HI - *Sac*II 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 *Bam*HI 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 *Stu*I site in the 3' non-coding sequence inserted into the *Bam*HI - *Sma*I 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**CGTCAAGAGCAAGTTC-3' to create the junction between an ATG codon at position 565 and the *Bam*HI 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**ATTTCATCAAGCA TTGTAC-3' to create the junction between an ATG codon at position 1017 and the *Bam*HI 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 *Xho*I - *Sac*II polylinker of pBluescript (Stratagene, La Jolla, CA) fused to *SUC2* by use of the following oligonucleotide primer: 5'-TCCCCGCGGCATCA**ATG**ACAAACGAAAC-3'. The *Sac*II site , 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 *Pst*I - *Not*I fragment of pPE4 was subcloned into pBluescript-SK+ (Stratagene). Oligonucleotide site-directed mutagenesis was used to insert a *Not*I site between amino acids 1892 and 1893 of *SEC16* (Kunkel *et al.*, 1987). The oligonucleotide sequence was 5'-CATCGCCTGCTATATATGCAGGCGGCCGCAGAACTCACCAAGCACATGC-3'. This plasmid was partially digested with *Not*I, and a 100 bp *Not*I - *Not*I 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.

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 ~1 x 10^8 cells/ml using 150 µCi/ml of ^{35}S-radiolabeled cysteine and methionine (NEN Expre^{35}S^{35}S, DuPont, Boston, MA). After 10 min, the chase was initiated by addition of cysteine and methionine to a final concentration of 30 µg/ml and 40 µg/ml, respectively. An aliquot of 2x10^7 cells was removed at time points and lysed using NaOH as described below for Western blots. The resulting extract was resuspended in 50 µl of 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. Carboxypeptidase Y 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 YPD to exponential phase (~1x10^7 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 approximately 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 a JEOL 1200CX electron microscope 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 *Pvu*II - *Pst*I 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 *E. 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 *Pvu*II - *Pst*I fragment of Sec16p employed 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 (~1x10^7 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 ~6 x 10^7 cells in 100 µl of medium and adding 17 µl of 1.85 M NaOH, 1 M β-mercaptoethanol for 10 min at 4°C (Yaífe 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, 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) 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). Western blots were developed using chemiluminescence (ECL kit, Amersham).

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 x 10^9 cells were suspended in 50 ml of 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 3700 U of lyticase in 10 ml of 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 of 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, 2μg/ml aprotinin, and 0.5 U/ml α_2-macroglobulin (all Boehringer Mannheim, Indianapolis, IN) (Jones, 1991). Cell lysis was complete as judged by light microscopy. Following lysis, the cell extract was subjected to a series of centrifugation steps using the TLA100 ultracentrifuge rotor (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 of 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 prior to fixation.

Cells were fixed for 1 h with 3.7% formaldehyde in the medium. A ten ml culture was collected by centrifugation and then spheroplasted with 100 U of 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 IgG-FITC antibody (Boehringer Mannheim). Samples mounted in medium containing DAPI and *p*-phenylenediamine were photographed with a Zeiss axioscope 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 of membranes, 2.4 mg of 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 of 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 TLA 100.3 rotor (Beckman 357448). 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 monoclonal antibody, a 1:1000 dilution of Sec22p polyclonal antibody (gift of Dr. Charles Barlowe, Dartmouth Medical School), or a 1:1000 dilution of Sec61p polyclonal antibody (gift of Dr. Randy Schekman, University of California, Berkeley). Protein detected by Western blotting was quantitated by densitometry using a LKB 2202 Ultroscan. 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, 2μg/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).

Vesicles formed *in vitro* from donor membranes prepared at 32,000 *g* were fractionated by gel filtration on a 14 ml (1 x 18 cm) Sephacryl S-1000 column (Pharmacia Biotech, 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 (Biorad).

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. Ten hours prior to 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 (Biorad).

Affinity Isolation of Sec16p and Sec23p

The full-length coding sequence of *SEC23* was fused to the glutathione-*S*-transferase gene (GST) (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). 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. $1x10^8$ 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 Biotech) 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 5x 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 *Bam*HI - *Sph*I 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 data base, 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 and Schekman, personal communication) and *sec16-4* and *sec16-5* were isolated as mutations synthetically lethal with *sec13-1* (Roberg and Kaiser, unpublished results). Each mutation was mapped by *in vivo* recombination tests with 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 was presumably 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.

The 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 carrying 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 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 carboxypeptidase Y (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 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 hours. 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 hr 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 (Figs. 4*b* and 4*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 temperature-sensitive 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 overproduction. Figure 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.

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 (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 weight 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 ten 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 monoclonal antibody

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 ten minutes with ^{35}S-methionine and cysteine, and extracts were prepared at times after addition of unlabeled amino acids. There was no reduction in labeled Sec16p after a 30 minute 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 1×10^4 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.

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 *g*, 10,000 *g*, 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) two hours after induction. Many, but not all, cells showed concentrated staining at the periphery of the DAPI stained nucleus (Fig. 8*a* and 8*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 throughout 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 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 uniform 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 prior to 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.

The 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* were each 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 glutathione *S*-transferase (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 monoclonal antibody. 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.

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 temperature 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 can not rigorously rule out the possibility that Sec16p is associated with vesicles derived from another source, such as the Golgi. 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 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 temperature-sensitive 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 can not 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-terminus, and the COOH-terminus of the protein. The five temperature-sensitive 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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Figure 1. *a* The predicted amino acid sequence of *SEC16*. The *SEC16* DNA sequence is available from GenBank/EMBL/DDBJ under accession number U23819. *b* Domain structure of Sec16p and locations of the temperature-sensitive 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.

a

```
   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  TDKNADVTCK 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
```

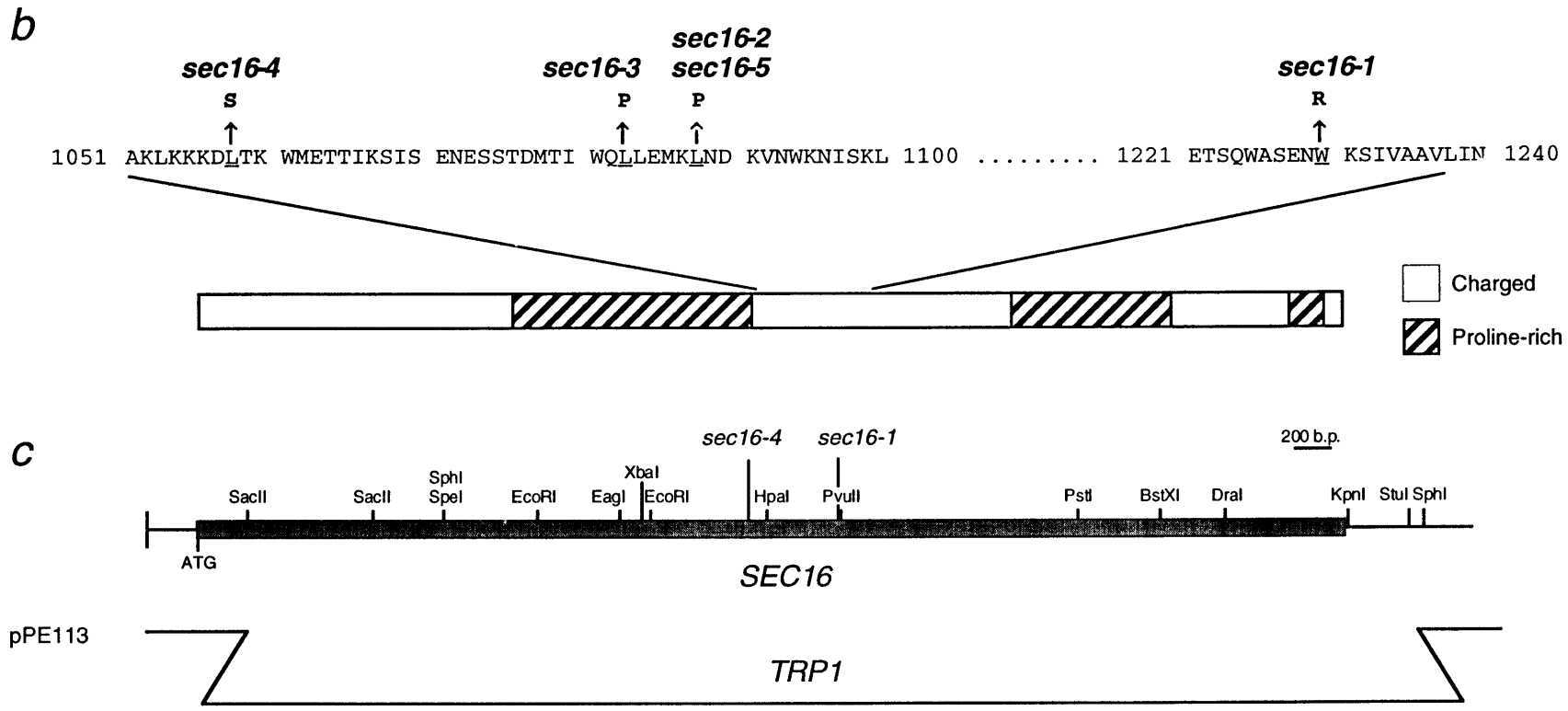
b
sec16-4
S
sec16-3
P
sec16-2
sec16-5
P
sec16-1
R
1051 AKLKKKDLTK WMETTIKSIS ENESSTDMTI WQLLEMKLND KVNWKNISKL 1100 ..... 1221 ETSQWASENW KSIVAAVLIN 1240
Charged
Proline-rich
c
sec16-4
sec16-1
200 b.p.
SacII
SacII
SphI
SpeI
EcoRI
XbaI
EagI
EcoRI
HpaI
PvuII
PstI
BstXI
DraI
KpnI
StuI
SphI
ATG
SEC16
pPE113
TRP1

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.

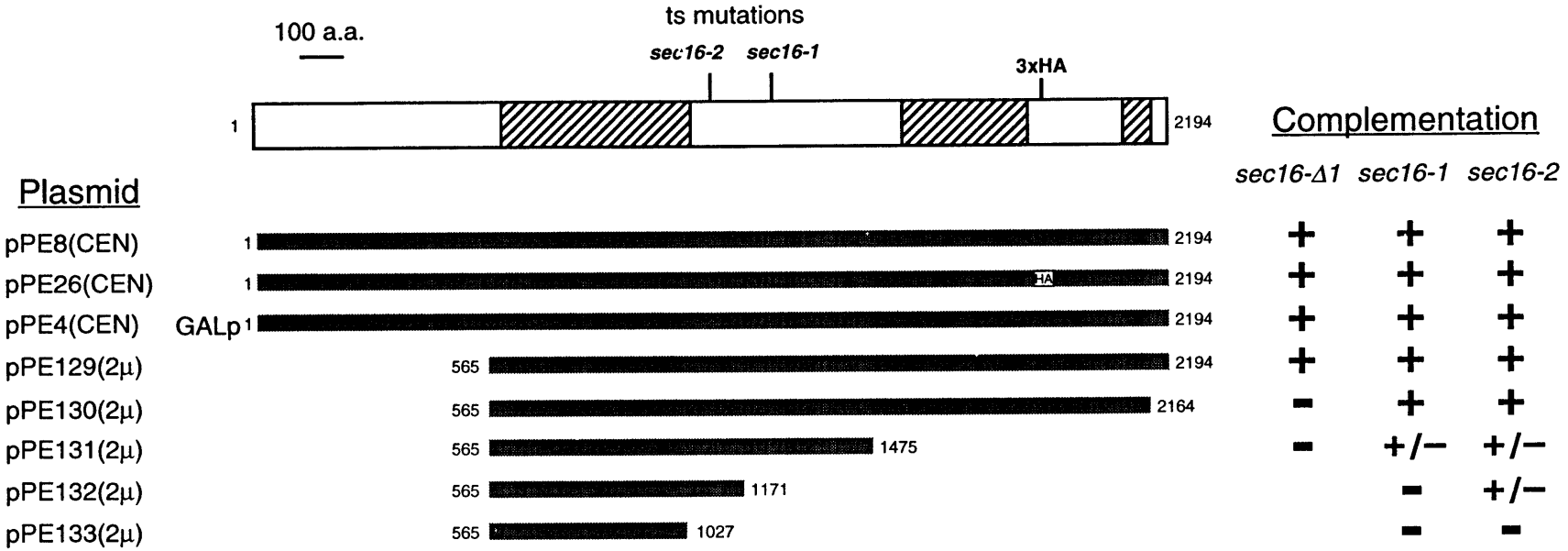
100 a.a.
ts mutations
sec16-2
sec16-1
3xHA
1
2194
Plasmid
Complementation
sec16-Δ1
sec16-1
sec16-2
pPE8(CEN)
1
2194
+
+
+
pPE26(CEN)
1
HA
2194
+
+
+
pPE4(CEN)
GALp
1
2194
+
+
+
pPE129(2μ)
565
2194
+
+
+
pPE130(2μ)
565
2164
-
+
+
pPE131(2μ)
565
1475
-
+/−
+/−
pPE132(2μ)
565
1171
-
+/−
pPE133(2μ)
565
1027
-
-

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 hours 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 hours (lanes 10-12). CKY50, a *sec16-2* ts strain, was grown at 37°C for one hour to express a secretion block (lanes 13-15). Cultures were labeled with ^{35}S trans-label 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).

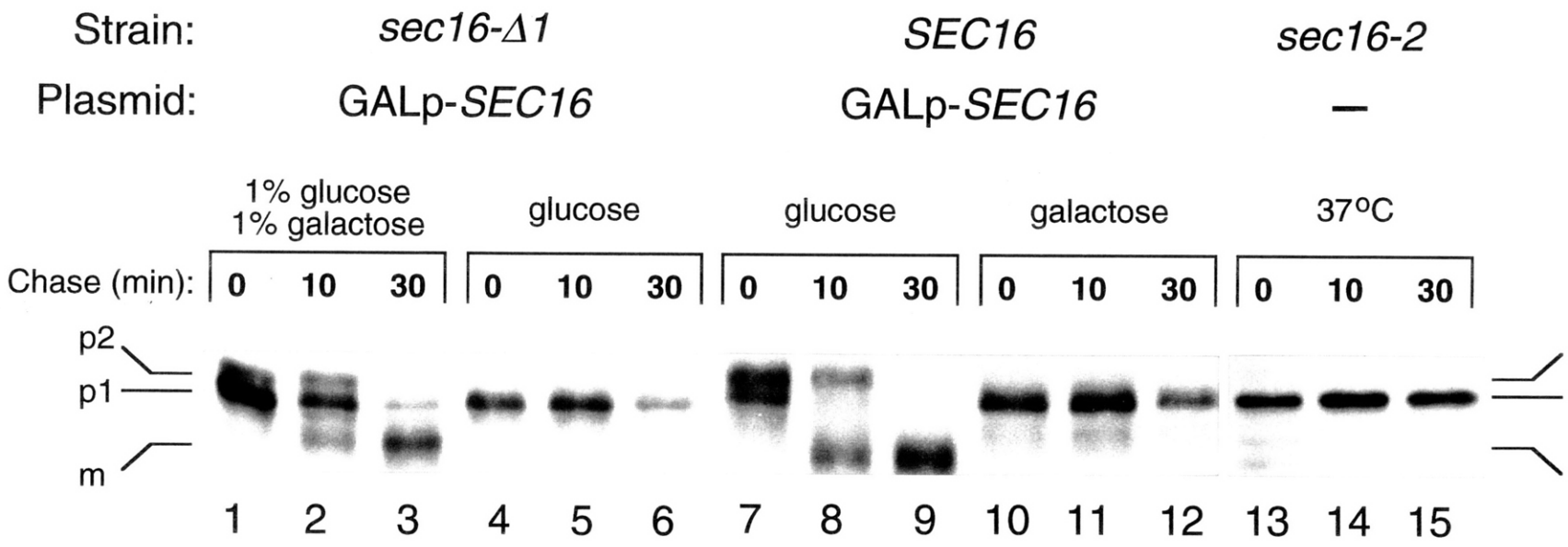
Strain:
sec16-Δ1
SEC16
sec16-2
Plasmid:
GALp-SEC16
GALp-SEC16
—
1% glucose
1% galactose
glucose
glucose
galactose
37°C
Chase (min):
0 10 30 0 10 30 0 10 30 0 10 30 0 10 30
p2
p1
m
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

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 one hour. *b* A *sec16-2* ts mutant (CKY50) grown at 25°C and shifted to 37°C for one hour. *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 hours at 30°C to deplete cells of Sec16p. Cells were fixed and stained with $KMnO_4$. Bar is 500 nm.

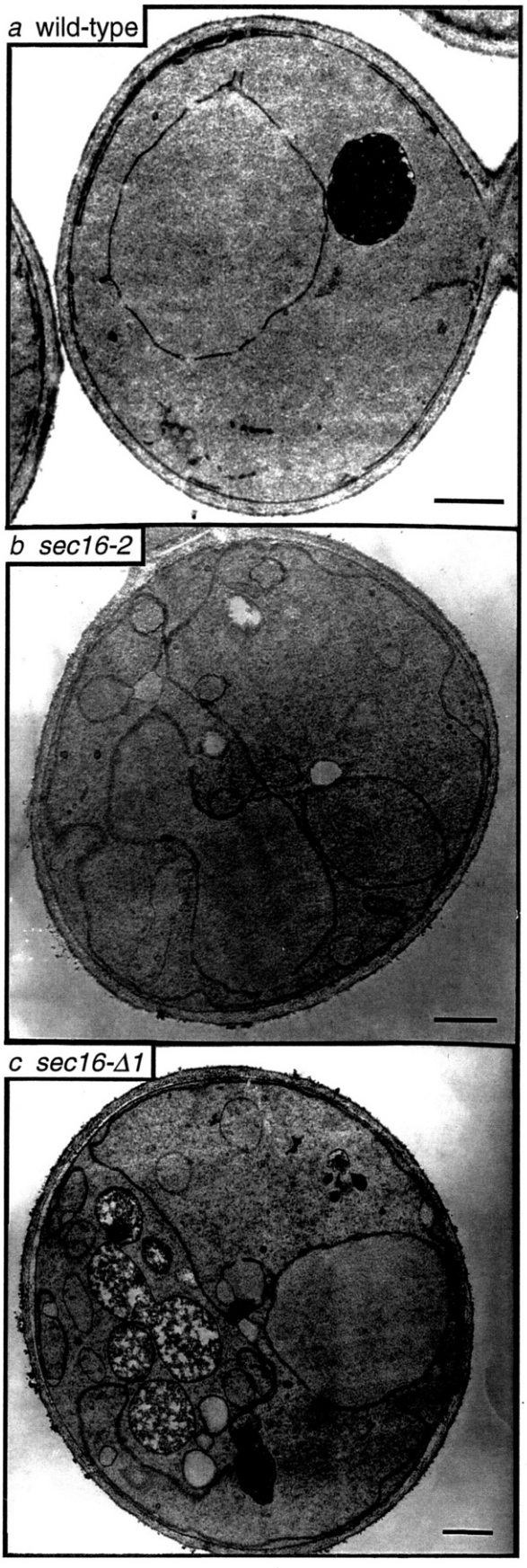
a wild-type
b sec16-2
c sec16-Δ1

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*.

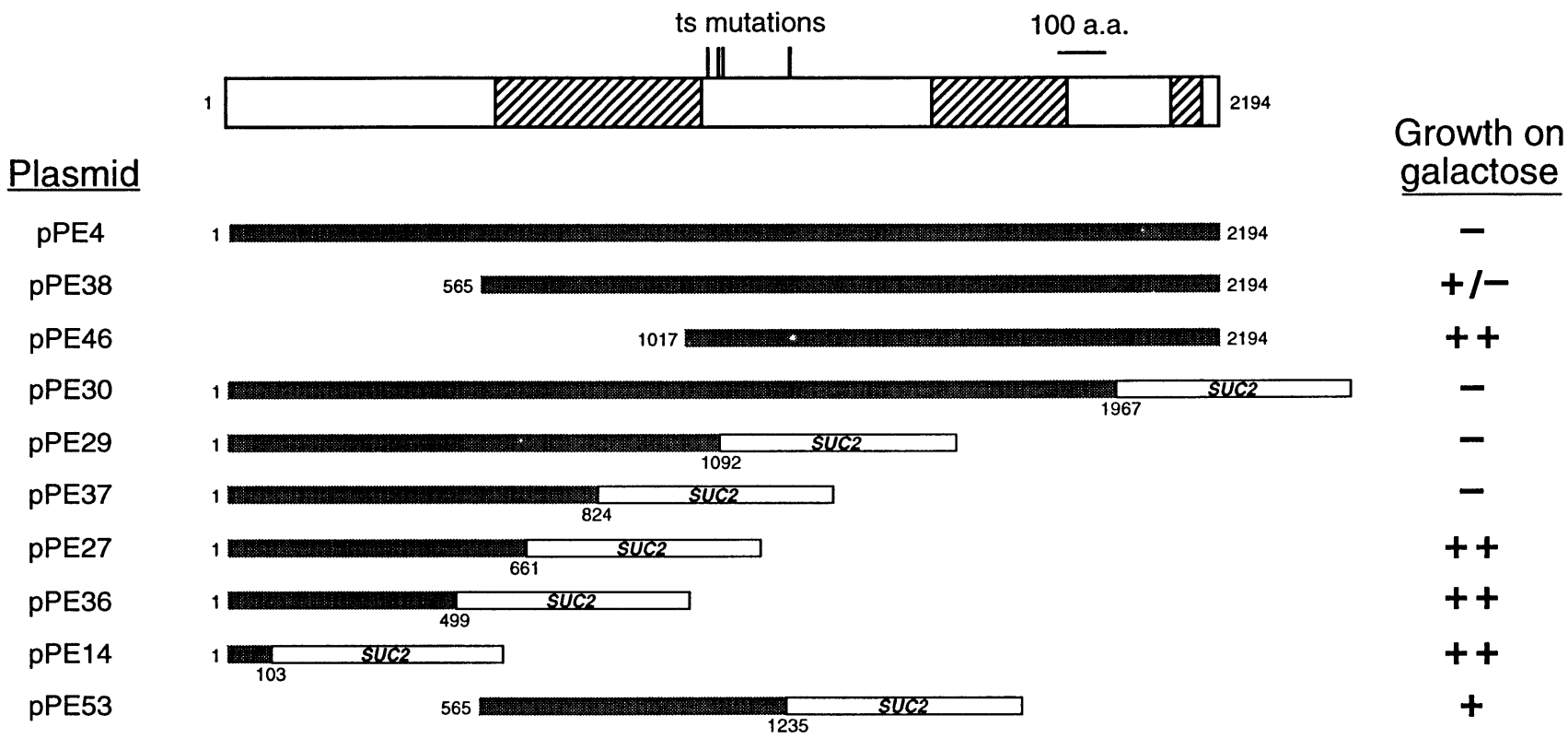
ts mutations
100 a.a.
1
2194
Plasmid
Growth on galactose
pPE4
1
2194
−
pPE38
565
2194
+/−
pPE46
1017
2194
++
pPE30
1
SUC2
1967
−
pPE29
1
SUC2
1092
−
pPE37
1
SUC2
824
−
pPE27
1
SUC2
661
++
pPE36
1
SUC2
499
++
pPE14
1
SUC2
103
++
pPE53
565
SUC2
1235
+

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 hours 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.

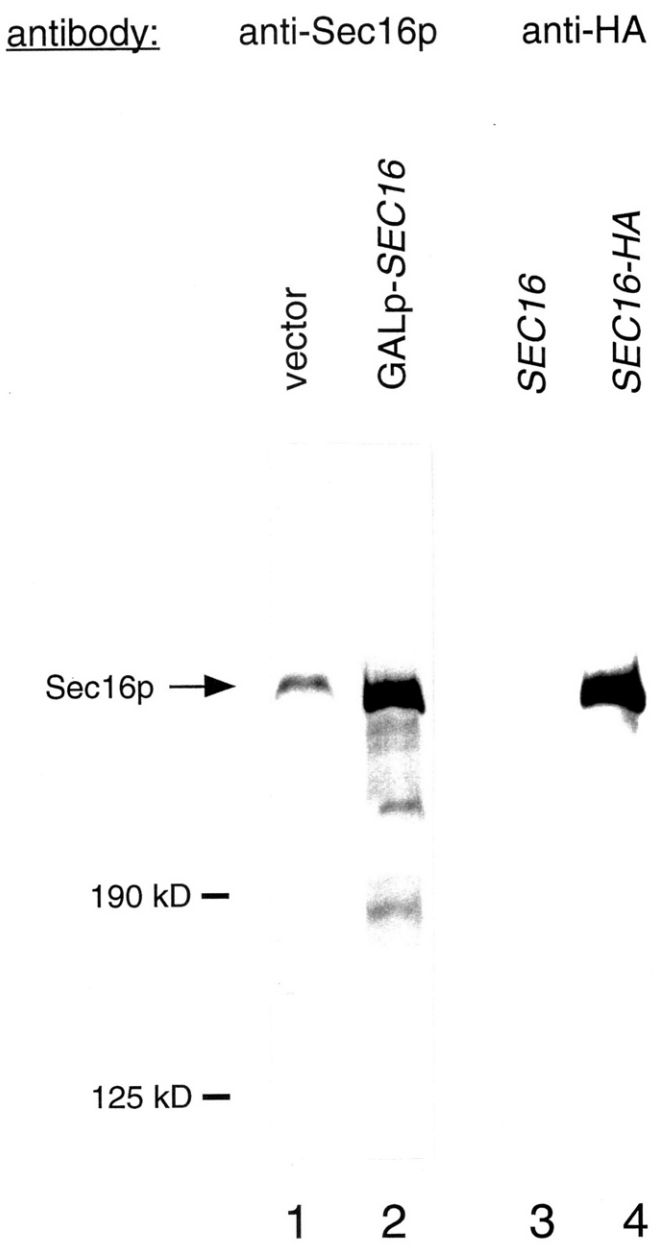
antibody:
anti-Sec16p
anti-HA
vector
GALp-SEC16
SEC16
SEC16-HA
Sec16p
190 kD
125 kD
1
2
3
4

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 *g*, 10,000 *g*, 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.

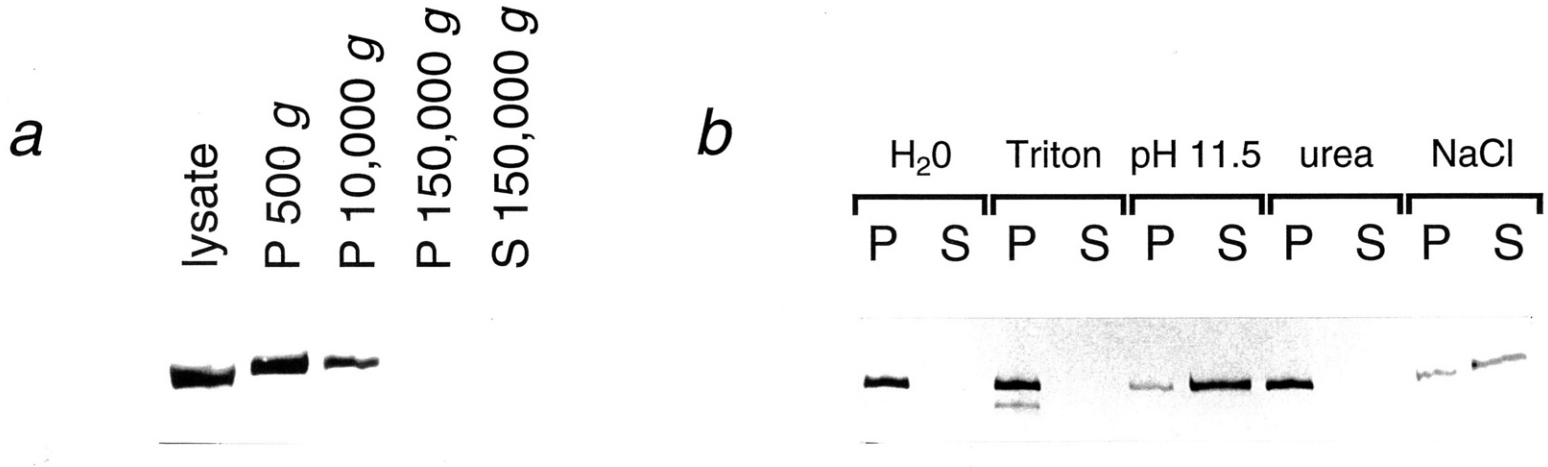
a
lysate
P 500 g
P 10,000 g
P 150,000 g
S 150,000 g
b
H20
Triton
pH 11.5
urea
NaCl
P S P S P S P S P S

Figure 8. Immunolocalization of Sec16p. A wild-type diploid strain, CKY241, expressing *SEC16* from the *GAL1* promoter was grown in galactose for 2 hours 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 DIC. Magnification: top panels, 2,800x; bottom panel, 1,760x.

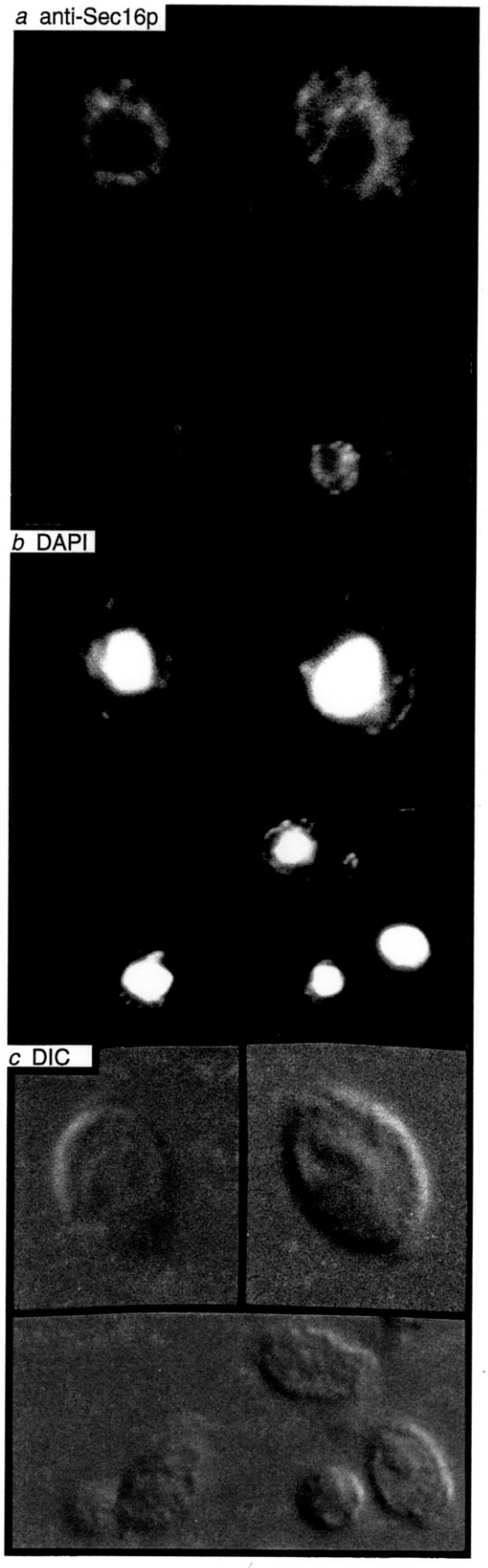
a anti-Sec16p
b DAPI
c DIC

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.

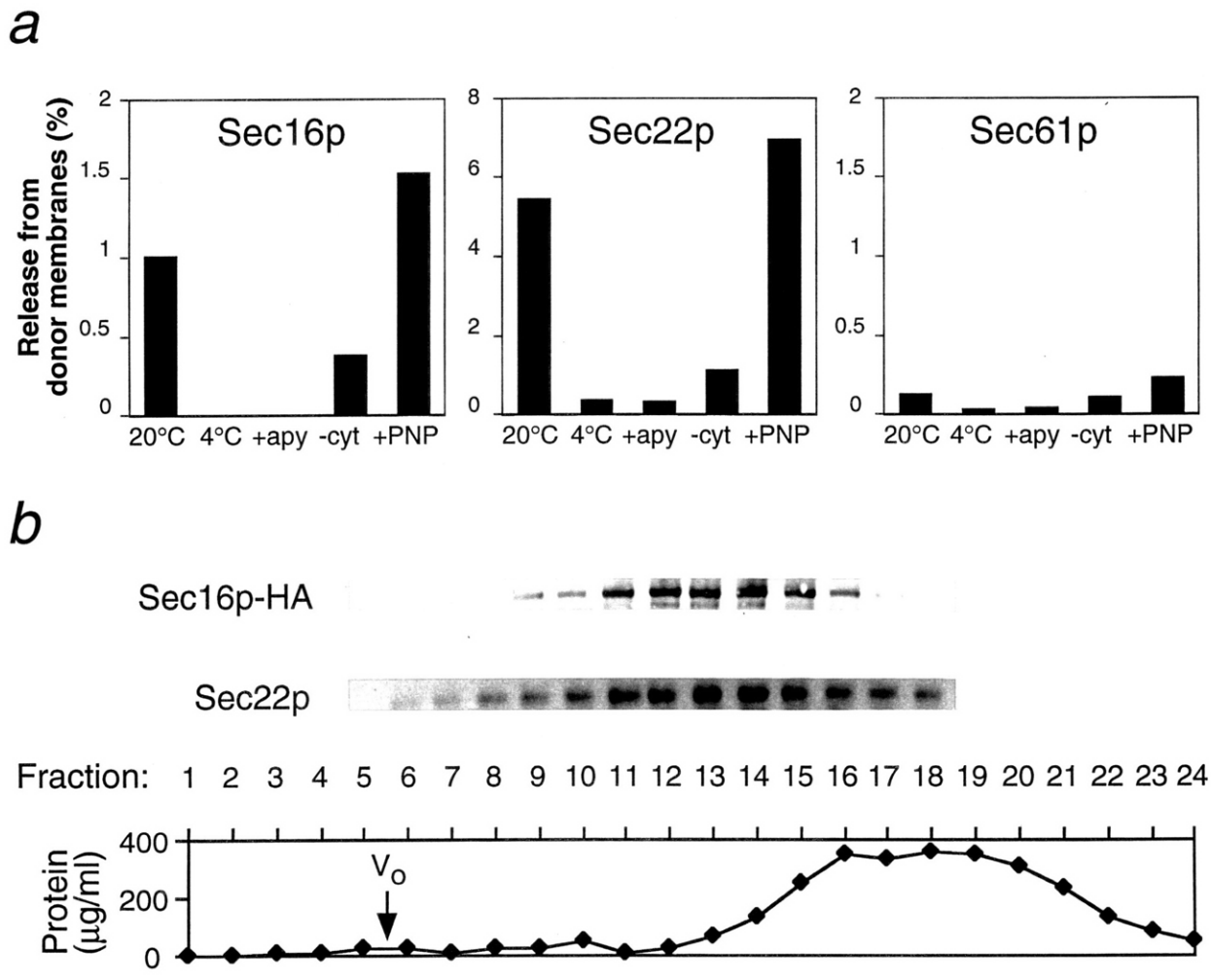
a
Release from donor membranes (%)
Sec16p
Sec22p
Sec61p
20°C 4°C +apy -cyt +PNP
b
Sec16p-HA
Sec22p
Fraction: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Protein (µg/ml)
400
200
0
Vo

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 $1x10^7$ 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 monoclonal antibody.

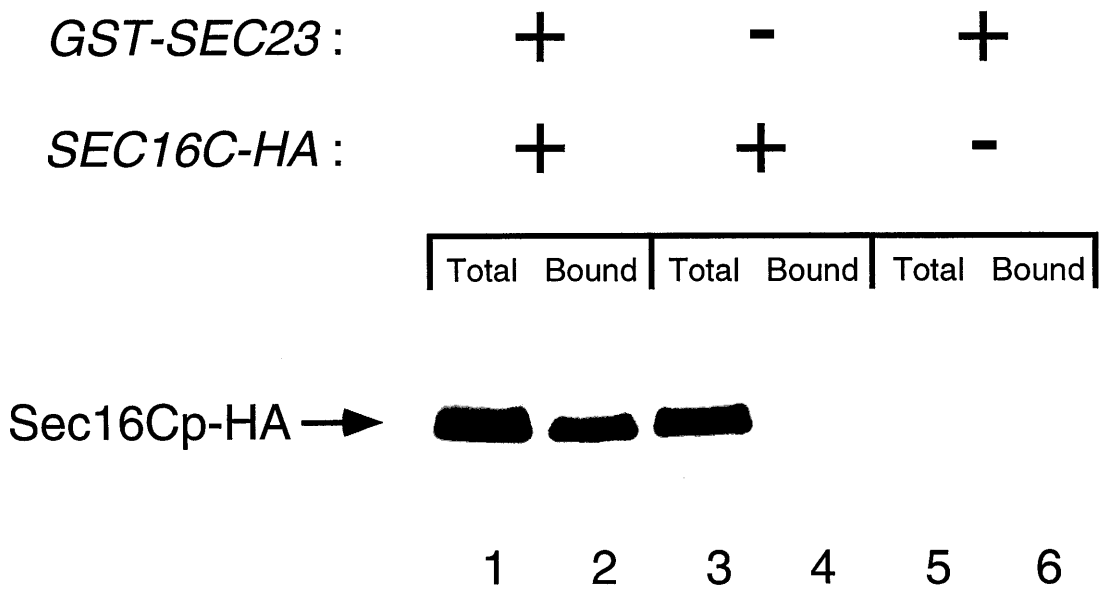
GST-SEC23 : + - +
SEC16C-HA : + + -
Total Bound Total Bound Total Bound
Sec16Cp-HA
1 2 3 4 5 6

Table I. *S. cerevisiae* strains

Strain	Genotype	Source
CKY8	*MATα ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY10	*MATa ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY19	*MATa/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	*MATa sec16-2 ura3-52*	Kaiser Lab Collection
CKY52	*MATa 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	*MATa sec16-3 ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY200	*MATa sec16-2 ura3-52 leu2-3,112 his4-619* Gal$^+$	Kaiser Lab Collection
CKY230	*MATa sec16-4 sec13-1 ura3-52 leu2-3,112 ade2 ade3* (pKR4)	Roberg and Kaiser, unpublished
CKY231	*MATa sec16-5 sec13-1 ura3-52 leu2-3,112 ade2 ade3* (pKR4)	Roberg and Kaiser, unpublished
CKY232	CKY96 (pPE4)	This study
CKY233	*MATa/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	*MATa 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	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 HIS4/ his4-619* Gal$^+$ (pPE4)	This study
CKY247	CKY237 (pCD43)	This study
CKY282	*MATa 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	Roberg and Kaiser, unpublished
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

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

Table III. Sec23p Interacts with the C-terminus of Sec16p by Two-Hybrid Assay

	ß-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 prior to the assay to induce expression of fusion proteins. Units of ß-galactosidase activity (nmol/mg x min) were calculated as (Optical density at 420 nm x volume of assay)/(0.0045 x concentration of protein in extract assayed x 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.

Chapter II - Appendix

Sec16p Is a Stable ER Protein

Preface

The initial characterization of the intracellular localization of Sec16p was performed using immunofluorescence microscopy on a diploid yeast strain that overexpressed Sec16p from the *GAL1* promoter. The development of a new polyclonal antibody recognizing the COOH-terminus of Sec16p allowed this analysis to be repeated in a diploid strain expressing Sec16p at wild-type levels. In addition, experiments demonstrating that Sec16p is a stable protein in vivo were described as "data not shown" in Chapter II. The results and methods of these experiments are contained in this appendix.

Materials and Methods

Construction of *SEC12-myc*

A version of *SEC12* tagged with the myc epitope recognized by the 9E10 mouse monoclonal antibody was generated to permit double labeling of yeast cells in immunofluorescence microscopy experiments. A PCR fragment made using oligonucleotide primers (5'-CAAAGGGATTACTTCTATGG-3', 5'-GCGAAT-TCAGCGGCCGCCAGCATCATCTATTTCTCG-3') and the plasmid pRH50 as template DNA was digested with SalI/EcoRI and inserted into pRH50 (Gimeno *et al.*, 1995). A cassette encoding three copies of the myc epitope flanked by NotI sites was inserted into a NotI site that was engineered at the COOH-terminus of Sec12p to yield pPE303, a 2μ plasmid containing *SEC12-myc* (Gimeno *et al.*, 1995). *SEC12-myc* was fully functional since it was able to support growth of a *sec12* deletion strain.

Sec16p Antiserum

Sec16p antiserum was elicited against a hybrid protein composed of Sec16p (aa 1892-2194) fused to glutathione *S*-transferase (Smith and Johnson, 1988). An expression vector for this fusion protein, pPE256, was constructed by digesting a PCR fragment generated using the oligonucleotide primers (5'-CGCGGATCCATGTACTCACCAAGTGTGG-3', 5'-CGGAATTCTATT-AGTGATGGTGATGGTGATGTTGTATGTTATCCATTA-3') and pPE10 (*SEC16-HA* under control of the *GAL1* promoter in pRS315) with BamHI/EcoRI and inserting this fragment into pGEX-2T (Smith and Johnson, 1988). The resulting fusion protein consisted of GST fused to a fragment of *SEC16* (aa 1892-2194) that contained a single influenza HA epitope at the NH_2-terminus of Sec16p and a repeat of six histidines at the COOH-terminus. This fusion protein was expressed in the *Escherichia coli* strain CKB175 (Gimeno *et al.*, 1996) and

purified first by affinity to glutathione Sepharose (Pharmacia Biotech, Piscataway, NJ) followed by purification using Ni-NTA resin (Qiagen Inc., Santa Clarita, CA) according to the manufacturer's instructions. Polyclonal antibodies were generated using two rabbits 826 and 827 as previously described (Covance Inc., Denver, PA; Griff *et al.*, 1992). An identical fusion protein lacking GST was expressed in the vector pET-21d (Novagen Inc., Madison, WI) and purified using Ni-NTA resin. Antibodies specific for Sec16p were affinity purified using the protein without GST as described (Griff *et al.*, 1992).

Indirect Immunofluorescence

The intracellular distribution of Sec16p and Sec12p-myc was examined in a diploid yeast strain PEY195 (a/α *leu2-3,112 ura3-52* Gal$^+$) transformed with a *SEC12-myc* plasmid, pPE303. The experiment was performed as previously described (Espenshade *et al.*, 1995) with the following exceptions. Yeast cells were grown at 30°C in synthetic media. Sec16p was detected using a 1:500 dilution of affinity purified 826 antibody (2.6 mg/ml). Sec12p-myc was detected using a 1:500 dilution of the 9E10 mAb (Gimeno *et al.*, 1995). Both primary antibodies were detected using a 1:100 dilution of either anti-rabbit or anti-mouse Ig-FITC antibody. Samples were photographed using T-Max 400 film (Eastman Kodak Co., Rochester, NY).

Sec16p Immunoprecipitation

To analyze the stability of Sec16p in vivo, a wild-type strain CKY8 (*MATα ura3-52 leu2-3,112*) was grown at 30°C to exponential phase in minimal medium. Cells were labeled for 30 min at a concentration of ~1 x 10^8 cells/ml using 150 μCi/ml of ^{35}S-radiolabeled cysteine and methionine (NEN Expre^{35}S^{35}S, DuPont, Boston, MA). After 30 min, the chase was initiated by

addition of cysteine and methionine to a final concentration of 30 μg/ml and 40 μg/ml, respectively. An aliquot of 8 x 10^7 cells was removed at the indicated time points. Extracts were prepared and the samples were processed as described previously (Espenshade *et al.*, 1995). Sec16p was isolated from each sample using 8 μg of affinity-purified Sec16p antiserum (rabbit 826) and Protein A-Sepharose (Pharmacia Biotech, Piscataway, NJ).

Results and Discussion

The intracellular distribution of Sec16p has been investigated previously using antibodies that recognized Sec16p (aa 1236-1695) in a diploid strain overexpressing Sec16p from the *GAL1* promoter (Espenshade *et al.*, 1995). Cells showed concentrated staining at the periphery of the nucleus typical of ER proteins as well as punctate staining dispersed throughout the cell. The generation of a second rabbit antiserum that recognizes the COOH-terminus of Sec16p, amino acids 1892-2194, provided an opportunity to re-examine the intracellular location in diploid cells expressing Sec16p at wild-type levels. The results with this antiserum were identical to those observed previously. Cells displayed bright staining at the periphery of the nucleus, but also showed punctate staining at other locations in the cell (Fig. 1). Antibodies to the resident ER protein, Sec12p-myc, also stained the nuclear periphery, but showed less of the punctate staining pattern seen with the Sec16p antibody. The location of the Sec16p punctate staining remains unknown. One possibility is that this staining pattern could represent ER to Golgi transport vesicles or perhaps Sec16p complexes on the ER membrane at the periphery of the cell. Collectively, these data support the conclusion that Sec16p is a peripheral ER membrane protein and establish Sec12p-myc as a useful marker for the ER in immunofluorescence microscopy experiments.

To investigate the stability of Sec16p in vivo, we performed a pulse-labelling experiment. Wild-type cells expressing Sec16p were labelled for 30 min followed by incubation with an excess of unlabelled amino acids. Sec16p was isolated by immunoprecipitation with Sec16p antiserum at several time points. Equivalent amounts of Sec16p were present at the 0 min and 60 min time points demonstrating that Sec16p is a stable protein with a half-life greater than 1 hr (Fig. 2).

Under these growth conditions, a cell doubles every 2.5 hr which requires the COPII-mediated transport of large quantities of membrane to the cell surface to generate the daughter yeast cell. The stability of Sec16p and the absence of a detectable cytosolic pool of Sec16p raise the question of what happens to Sec16p after it is part of the COPII vesicle coat. Sec16p may dissociate from the vesicle prior to fusion and rapidly reassociate with the ER membrane to participate in future rounds of vesicle formation. Alternatively, Sec16p may be recycled back to the ER on Golgi to ER retrograde vesicles. However, Sec16p would be expected to function only as cargo since Sec16p is not required for the formation of these vesicles (Letourneur *et al.*, 1994). A third possibility is that Sec16p could function in additional vesicle mediated transport steps later in the secretory pathway. Experiments involving higher resolution localization studies are in progress to try to address these questions.

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Figure 1. Immunolocalization of Sec16p. A wild-type diploid strain, PEY195, expressing *SEC12-myc* was examined by indirect immunofluorescence using either affinity-purified rabbit anti-Sec16p antibody or anti-myc 9E10 mAb and the appropriate fluorescein-conjugated secondary antibody. DAPI-stained nuclear DNA was also visualized. *Top panels*, localization of Sec16p. *Bottom panels*, localization of Sec12p-myc.

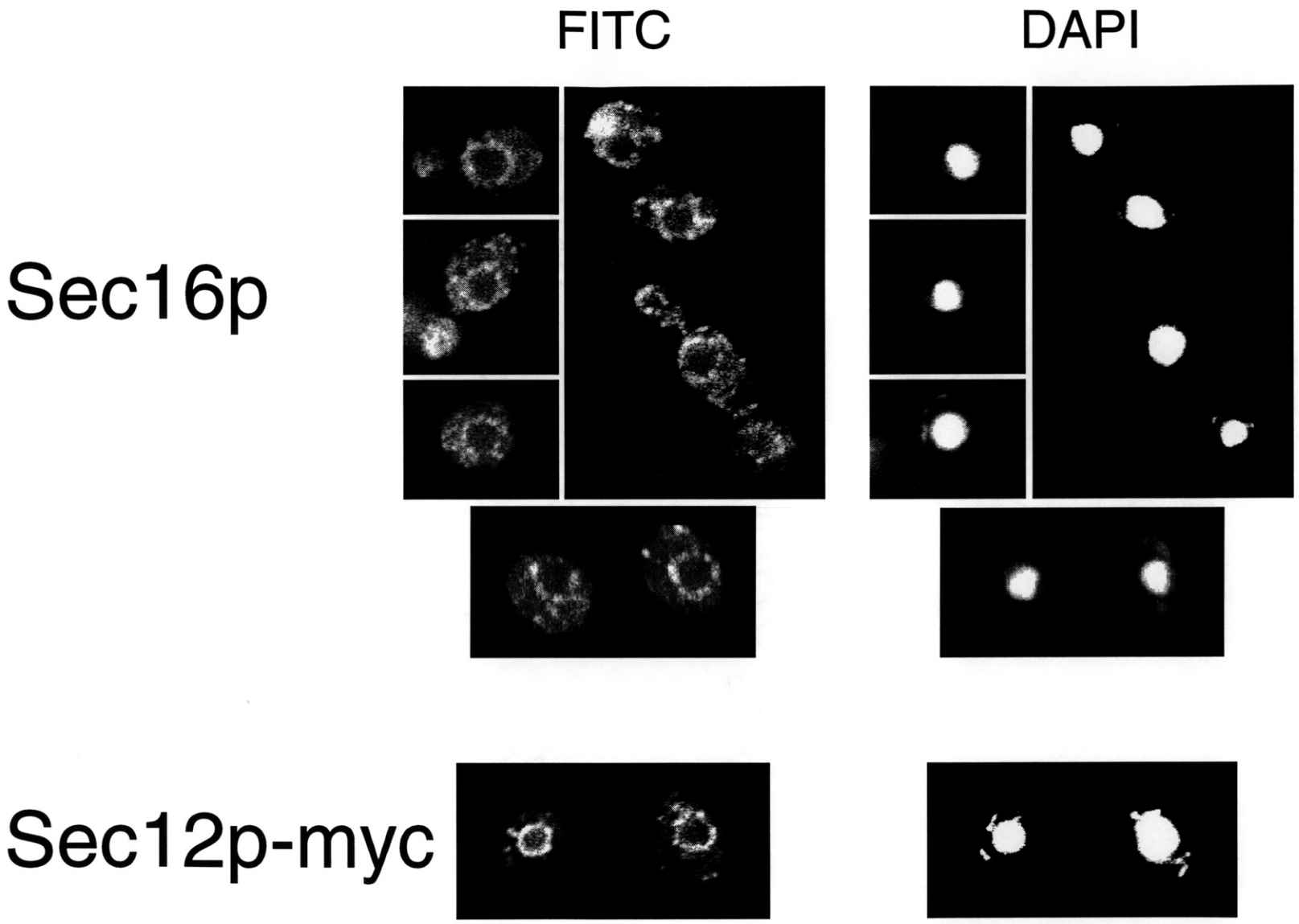
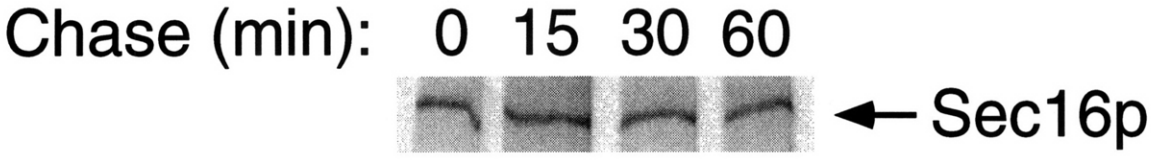

FITC
DAPI
Sec16p
Sec12p-myc

Figure 2. Sec16p is a stable protein. A wild-type yeast strain, CKY8, was grown in glucose at 30˚C and cells were labeled for 30 min with ^{35}S-translabel and chased by the addition of excess unlabeled methionine and cysteine for 15, 30, or 60 min. Sec16p was immunoprecipitated from labeled extracts and resolved by SDS-PAGE.


Chase (min): 0 15 30 60
Sec16p

Chapter III

COPII Coat Subunit Interactions: Sec24p and Sec23p Bind to Adjacent Regions of Sec16p

Preface

This chapter has been published in Molecular Biology of the Cell as: Gimeno, R.E., P. Espenshade, and C.A. Kaiser. 1996. COPII Coat Subunit Interactions: Sec24p and Sec23p Bind to Adjacent Regions of Sec16p. *Mol. Biol. Cell.* 7:1815-1823.

This chapter represents a collaboration between myself and Ruth Gimeno. Ruth Gimeno performed the two-hybrid assay experiments and the binding studies in yeast cell extracts.

Abstract

Formation of COPII coated vesicles at the endoplasmic reticulum (ER) requires assembly onto the membrane of five cytosolic coat proteins, Sec23p, Sec24p, Sec13p, Sec31p and Sar1p. A sixth vesicle-coat component, Sec16p, is tightly associated with the ER membrane, and has been proposed to act as a scaffold for membrane association of the soluble coat proteins. We previously showed that Sec23p binds to the COOH-terminal region of Sec16p. Here we use two-hybrid and coprecipitation assays to demonstrate that the essential COPII protein Sec24p binds to the central region of Sec16p. In vitro reconstitution of binding with purified recombinant proteins demonstrates that the interaction of Sec24p with the central domain of Sec16p does not depend on the presence of Sec23p. However, Sec23p facilitates binding of Sec24p to Sec16p and the three proteins can form a ternary complex in vitro. Truncations of Sec24p demonstrate that the NH_2-terminal and COOH-terminal regions of Sec24p display different binding specificities. The COOH-terminus binds to the central domain of Sec16p while the NH_2-terminus of Sec24p binds to both the central domain of Sec16p and to Sec23p. These findings define binding to Sec16p as a new function for Sec24p and support the idea that Sec16p is a scaffold molecule.

Introduction

Transport of proteins between compartments of the secretory pathway is mediated by cytoplasmic vesicles that form by budding from the membrane of the donor compartment. In the best studied cases, vesicle formation is driven by the assembly of vesicle coats that are composed of particular sets of peripheral membrane proteins (reviewed by Schekman and Orci, 1996). COPII coated vesicles mediate transport from the endoplasmic reticulum (ER) to the Golgi apparatus in both yeast and mammalian cells (Barlowe *et al.*, 1994; Schekman and Orci, 1996). Genetic and biochemical studies in yeast have identified six proteins that are components of the COPII coat and that are required for vesicle formation in vivo and in vitro (Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990; Hicke *et al.*, 1989; Hicke *et al.*, 1992; Salama *et al.*, 1993; Barlowe *et al.*, 1994). Five of these proteins - two heteromeric protein complexes, Sec13p/Sec31p and Sec23p/Sec24p, and a small GTP-binding protein, Sar1p - are found in the cytosol and in association with membranes. Vesicle formation can be reconstituted by adding these five proteins in soluble form to ER membranes that have been extracted of peripheral proteins with urea (Barlowe *et al.*, 1994). The sixth essential COPII protein, Sec16p, is a 240 kD peripheral membrane protein that is found on membranes but not in soluble form and resists extraction by urea. In an in vitro budding reaction, Sec16p is incorporated into the vesicle coat starting as a peripheral ER protein not as a soluble protein (Espenshade *et al.*, 1995).

Clearly, an important aspect in COPII vesicle formation is the recruitment of cytosolic coat components to the membrane. Drawing an analogy to the role of the small GTP-binding protein ARF in recruitment of the coatomer (COPI) coat to Golgi vesicles (Donaldson *et al.*, 1992), the small GTP-binding protein Sar1p has been proposed to regulate recruitment of Sec23p/Sec24p and

Sec13p/Sec31p to the ER membrane (Schekman and Orci, 1996). Membrane-associated receptors for small GTP-binding proteins and coat components have been suggested to participate in the recruitment of clathrin coats and COPI coats to the Golgi membrane (Helms *et al.*, 1993; Stamnes *et al.*, 1993; Traub *et al.*, 1993). For COPII coats, Sec16p appears to have the properties of a membrane receptor for coat formation since Sec16p is tightly bound to the membrane and associates with the soluble COPII coat component Sec23p (Espenshade *et al.*, 1995). Moreover, genetic interactions between *SEC16* and *SAR1* suggest that assembly of Sec16p with soluble coat proteins may be regulated by Sar1p (Gimeno *et al.*, 1995).

We became interested in a possible interaction between Sec24p and Sec16p, when we identified a Sec24p homologue in a two-hybrid screen for proteins that bind to the central domain of Sec16p (R. E. Gimeno and C. Kaiser, unpublished). Sec24p is a 105 kD protein that is required for vesicle budding in vitro (Hicke *et al.*, 1992), but its precise role in vesicle formation has not been established. Sec24p copurifies with Sec23p as part of a 300-400 kD complex (Hicke *et al.*, 1992). Sec23p stimulates the GTPase activity of Sar1p in vitro, but Sec24p does not appear to contribute to this activity (Yoshihisa *et al.*, 1993).

In this paper, we show that Sec24p binds to the central region of Sec16p, a portion of Sec16p molecule that is separable from the region at the COOH-terminus of Sec16p that is required for binding to Sec23p. The identification of a second COPII coat component as a Sec16p binding protein reinforces the proposal that Sec16p is a scaffold that organizes the COPII coat.

Materials and Methods

General Techniques

Yeast transformations were performed using standard methods (Kaiser *et al.*, 1994). DNA manipulations were carried out as described (Sambrook *et al.*, 1989). For PCR, Vent polymerase was used (New England Biolabs, Beverly, MA). Western blotting was performed as described (Gimeno *et al.*, 1995) using the following antibodies: anti-invertase (1/1,000), anti-Sec23p (1/500; kindly provided by L. Hicke), anti-Sec24p (1/1,000; kindly provided by T. Yoshihisa), anti-GST (1/1,000; Santa Cruz Biotechnology, Santa Cruz, CA), and HRP-coupled sheep anti rabbit Ig (1/10,000; Amersham Corp., Arlington Heights, IL). Unless otherwise noted, protein concentrations were determined by the Biorad Protein Assay (Biorad Laboratories, Hercules, CA) using BSA (Boehringer Mannheim, Indianapolis, IN) as a standard.

Isolation of *sec24-1* and Cloning of *SEC24*

A temperature-sensitive allele of *SEC24* was isolated by screening a collection of 1800 random temperature-sensitive mutants for accumulation of the ER forms of carboxypeptidase Y and invertase by Western blotting (E. Chitouras, A. Frand, and C. A. Kaiser, unpublished). One of these mutants, EH382, displayed a strong, temperature-dependent accumulation of the ER-forms of CPY and invertase. This secretion defect segregated 2:2 in crosses and cosegregated with the temperature-sensitive growth defect, indicating that both growth and secretion defects were caused by a single mutation. The gene responsible for the defects was cloned by complementation of the temperature-sensitive growth defect of EH382 using a library of yeast genomic DNA in pCT3 (Thompson *et al.*, 1993). One complementing clone, pAF70, contained a 4.3 kb insert. Comparison of the restriction map and DNA sequence of pAF70 with the

sequence of *SEC24* (generously supplied by T. Yoshihisa and R. Schekman) showed that pAF70 contained the entire *SEC24* open reading frame flanked by 0.8 kb at the 5'- and 3'- end each. To demonstrate that the mutation in EH382 was indeed a mutation in *SEC24*, the chromosomal *SEC24*-locus of a wild-type strain was marked with *URA3* by directing integration of the plasmid pRH285, containing a 3 kb BamHI fragment with the COOH-terminal 896 amino acids of *SEC24*, to the homologous chromosomal locus by cleaving pRH285 in the insert sequences with *Bgl*II before transformation. The marked strain was then crossed to EH382. Tetrad analysis showed complete linkage of the plasmid sequences to the Ts growth phenotype. The *SEC24* allele in EH382 was designated *sec24-1*.

Two-Hybrid Protein-Protein Interaction Assay

Protein-protein interactions were tested in the two-hybrid system as described (Gyuris *et al.*, 1993; Bartel and Fields, 1995). Interactions were tested between proteins fused to the *lexA* DNA-binding domain that were expressed from either the constitutive *ADH* promoter or the inducible *GAL1* promoter and proteins fused to either the acidic activation domain of *GAL4* expressed from the *ADH* promoter or to an acidic bacterial sequence expressed from the *GAL1* promoter. All interactions were tested by measuring the expression of β-galactosidase from the reporter construct pSH18-34 in EGY40 (Golemis and Brent, 1992).

Plasmids were constructed as follows: A *SEC24* fusion to *lexA* lacking the NH_2-terminal 33 amino acids (pRH286) was constructed by inserting the 3 kb *BamH*I fragment of pAF70 into pEG202. To create a full-length *SEC24* fusion to *lexA* (pRH287), the NH_2-terminus of *SEC24* was amplified by PCR and ligated into pEG202, creating pRH284. The COOH-terminus of *SEC24* was

then inserted by ligating the 3 kb *BamH*I fragment of pAF70 into pRH284. The NH_2-terminal *SEC24 lexA* fusion (pRH321) contains the 2 kb *EcoR*I fragment of pRH287 ligated into pEG202. The fusion of the COOH-terminal region of *SEC24* to *lexA* (pRH347) was made by first inserting the 0.6 kb *EcoR*I fragment of pRH287 into pEG202-AAT, a derivative of pEG202, and then inserting the 1.1 kb *Apa*I-*Xho*I fragment of pRH286 into the resulting construct. To make a *GAL1* promoted *SEC23* fusion to *lexA* (pDS79), the *EcoR*I-*Xho*I fragment of pPE82 (Espenshade *et al.*, 1995) was inserted into pGILDA, a *GAL1* promoted derivative of pEG202 (kindly provided by D. Shaywitz). The central domain of Sec16p (aa 565-1235) was fused to the *GAL4* acidic activation domain by inserting the *BamH*I fragment of pPE53 (Espenshade *et al.*, 1995) into pGAD-GH (Hannon *et al.*, 1993) to make pPE167. A *lexA*-fusion containing aa 447-1235 of Sec16p (pPE62) was made by inserting the *Nco*I-*Pvu*II fragment of pPE4 (Espenshade *et al.*, 1995) into pEG202.

Indicator cells were grown to exponential phase in selective medium containing 2% glucose. To measure interactions of proteins expressed from the *GAL1* promoter, cells were grown to exponential phase in selective medium containing 2% raffinose, then galactose was added to 2% and growth was continued for an additional 5 h. β-galactosidase activity assays were performed on glass-bead extracts as described (Kaiser *et al.*, 1994). Units of β-galactosidase are expressed as: [OD_{420} x vol. of assay] / [0.0045 x protein concentration in extract x vol. of extract assayed x time].

Coprecipitation of GST-fusion Proteins from Yeast Extracts

Glutathione-S-transferase (GST) fusions for coprecipitation experiments were constructed as follows. *SEC24* (aa 34-926; pRH305) was fused to the GST gene expressed from the *GAL1* promoter by inserting a 3 kb *BamH*I

fragment of pAF70 into pPE127, a derivative of pRD56 (Espenshade *et al.*, 1995). To construct a GST fusion to the NH_2-terminus of *SEC24* (pRH348; aa 34-666), a 2 kb *BamH*I-*EcoR*I fragment of pRH321 was inserted into pPE127. A GST-fusion to the COOH-terminus of *SEC24* (pRH360; aa 666-926) was created by first inserting the 0.6 kb *EcoR*I fragment of pRH286 into pPE127, then cutting the resulting plasmid with *Bgl*II and *Xho*I and ligating it with the 1 kb *Bgl*II-*Xho*I fragment of pRH305. To produce a soluble and detectable central domain of Sec16p (pRH317), invertase was fused to amino acids 565-1235 of Sec16p, and the fusion protein was expressed from the *GAL1* promoter. pRH317 is identical to pPE53 (Espenshade *et al.*, 1995), with the exception that the vector backbone is marked with *LEU2*. To facilitate detection of Sec23p, *SEC23* was expressed from the *GAL1* promoter (pPE123).

Coprecipitation experiments were performed in CKY473 (*Matα*, *ura3-52, leu2-3,112*, Gal+). Cells were grown to exponential phase in selective medium containing 2% raffinose, galactose was added to 2%, and extracts were prepared 3 to 4 h later. 10-16 x 10^7 cells (5-8 OD_{600} units) were suspended in 30 µl CoIP buffer (20 mM HEPES, pH 6.8, 80 mM potassium acetate, 0.2 M NaCl, 5 mM magnesium acetate, 0.02 % Triton X-100, 5 mM DTT), containing a protease inhibitor cocktail (2.5 x 10^{-4} U/ml α_2-macroglobulin, 1 mM PMSF, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, and 2 µg/ml aprotinin; all Boehringer Mannheim). Cells were lysed by vigorous agitation with glass beads 4 x 20 sec with 1 min intervals on ice. Extracts were diluted with 200 µl CoIP buffer as above, and the lysate was cleared by centrifugation at 13,000 x *g* for 10 min followed by centrifugation at 100,000 x *g* for 30 min. The supernatant was diluted to 700 µl with CoIP buffer (supplemented as above), glutathione Sepharose 4B beads (Pharmacia, Piscataway, NJ) were added, and samples were incubated for 1 h at 25°C. The beads were washed 4 times with CoIP

buffer and bound proteins were released by boiling in 30 µl ESB (60 mM Tris HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.001% bromophenol blue).

Preparation of Recombinant Sec23p and Sec24p

Sec23p and Sec24p were expressed as GST-fusion proteins in *E. coli* strains AP401 (*lon*::*ΔTn10*; Pakula, 1988) and CKB175 (*ompT*$^-$, *lon*::*ΔTn10*; kindly provided by T. Opperman, M.I.T.), respectively. To construct the Sec23p expression vector (pPE124), the *BamH*I-*Xho*I (filled in) fragment of pPE119 (Espenshade *et al.*, 1995) was ligated into pGEX-2T (Pharmacia). A fusion of *SEC24* to GST (pPE166) was made by inserting the *Sma*I fragment of pRH325 into the *Sma*I site of pGEX-2T. pRH325 contains the entire *SEC24* reading frame with the NH_2-terminal 1.2 kb derived from pRH287 and the COOH-terminal 1.6 kb derived from pRH305.

Fusion proteins were induced and purified as described (Smith, 1993) with the following modifications. Cultures were grown to exponential phase at 30°C for GST-Sec23p and at 25°C for GST-Sec24p and fusion proteins were induced by addition of 0.5 mM IPTG and growth was continued for 2-3 h. Cells were lysed by sonication in phosphate-buffered saline (PBS) containing 0.5 mM EDTA and the same protease inhibitor cocktail used for the coprecipitation experiments. After addition of 1% Triton X-100, cell debris was removed by centrifugation at 16,000 x *g* for 10 min at 4°C. Fusion proteins were isolated by batch purification using glutathione Sepharose 4B beads (Pharmacia). Sec23p and Sec24p were liberated from the GST moieties by cleavage of the bound fusion proteins with thrombin (Sigma, St. Louis, MO) in cleavage buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM $CaCl_2$). Cleavage was stopped by addition of 1 mM PMSF. Recombinant proteins were stored in 10% glycerol at -80°C. Protein concentration was determined by comparison of purified protein

to dilutions of bovine serum albumin, Fraction V (Boehringer Mannheim) after resolution by SDS-PAGE and staining with Coomassie brilliant blue (BRL Life Technologies, Gaithersburg, MD).

Binding of recombinant Sec24p to recombinant Sec23p was assayed as follows. 250 µl bacterial lysate containing GST-Sec23p (in PBS, 0.02% Triton X-100, total protein: 0.5 mg) was incubated with 30 µl packed glutathione beads for 1 h at 4°C. Beads were collected by centrifugation and washed two times with 1 ml of PBS, 0.02% Triton X-100 and once with 1 ml of binding buffer (25 mM Hepes-KOH pH 6.8, 0.1% Triton X-100, 1 mM $MgCl_2$, 0.5 mM DTT, 0.25 mg/ml BSA). Washed beads contained ~11 pmol GST-Sec23p, as judged by Western blotting using GST as a standard. Beads were resuspended in 35 µl binding buffer, and 6 pmol Sec24p in 15 µl cleavage buffer was added. As a control, Sec24p was added to 30 µl packed glutathione beads not preincubated with GST-Sec23p. Reactions were incubated at 22°C for 1 h, the beads were collected by centrifugation, and the supernatant containing unbound Sec24p was mixed with an equal volume of 2 x ESB. Beads were washed two times with 1 ml of binding buffer and solubilized by addition of 100 µl of 1 x ESB. Equal volumes of protein samples were separated by SDS-PAGE and visualized by staining with Coomassie brilliant blue (BRL).

Preparation of Yeast GST-Sec16 Fusion Proteins and In Vitro Binding Assays

The central and COOH-terminal domain of Sec16p (amino acids 565-1235 and 1645-2194, respectively) were expressed in yeast as GST-fusion proteins from the *GAL1* promoter (pRH323 and pPE122, respectively). pRH323 is the 2 kb *BamH*I fragment of pPE53 (Espenshade *et al.*, 1995) in pPE127. pPE122 has been described (Gimeno *et al.*, 1995). As a control, GST (no

fusion) was expressed from the *GAL1* promoter (pRD56; Espenshade *et al.*, 1995).

GST-fusion proteins were expressed in CKY474 (*MATα leu2-3,112 ura3-52 pep4::LEU2* Gal+). Cells were grown to exponential phase in selective medium containing 2% raffinose, galactose was then added to 2%, and cells were grown for an additional 4 hours at 30°C. Cells were spheroplasted as described (Espenshade *et al.*, 1995) and gently lysed with glass beads in lysis buffer (20 mM Hepes-KOH, pH 6.8, 80 mM KOAc, 5 mM MgOAc, 0.02% Triton X-100, 0.6 M NaCl), containing the protease inhibitor cocktail as above. Cell debris was removed by centrifugation of the lysate at 17,500 x *g* for 15 min at 4°C. The supernatant was frozen in liquid nitrogen and stored at -80°C.

For binding experiments, aliquots of the frozen supernatants were mixed with 30 µl of packed glutathione sepharose beads (Pharmacia) in a total volume of 500 µl lysis buffer. Beads were incubated for 1 h at 4°C and then collected by centrifugation. The supernatant was discarded and the beads were washed two times with 1 ml lysis buffer, once with lysis buffer lacking NaCl (20 mM Hepes-KOH, pH 6.8, 80 mM KOAc, 5 mM MgOAc, 0.02% Triton X-100) and once with binding buffer (25 mM Hepes-KOH pH 6.8, 0.1% Triton X-100, 1 mM $MgCl_2$, 0.5 mM DTT, 0.25 mg/ml BSA). Beads were resuspended in 35 µl of binding buffer to which the indicated amount of recombinant Sec23p or Sec24p in 15 µl of cleavage buffer was added. Unless otherwise indicated, the final concentration of NaCl in the reaction was 45 mM. Reactions were incubated for 1 h at 22°C, beads were then collected by centrifugation and unbound recombinant Sec24p or Sec23p was sampled by mixing 20 µl of supernatant with an equal volume of 2 x ESB. Beads were washed two times with 1 ml of binding buffer and bound proteins were solubilized by addition of 100 µl of 1 x ESB (GST and GST-Sec16p central domain). Samples that contained GST

fused to the COOH-terminal domain of Sec16p were first treated with 0.1 μg thrombin in 50 μl cleavage buffer for 1 h and then solubilized by adding an equal volume of 2 x ESB. Thrombin digestion was necessary to distinguish Sec24p from GST-Sec16p (aa 1638-2194), since these two proteins migrated similarly on gels and the anti-Sec24p antibody crossreacted with the GST-fusion protein. Equal volumes of bound and unbound proteins were resolved by SDS-PAGE and analyzed by Western blotting.

Results

Sec24p Binds to the Central Domain of Sec16p in the Two-Hybrid System

Binding of Sec24p to Sec16p was first examined using the yeast two-hybrid system (Fields and Song, 1989; Gyuris *et al.*, 1993). Interaction between two proteins, one fused to the *lexA* DNA-binding domain and the other fused to an acidic transcription activation domain, was detected by the level of β-galactosidase expression in yeast from a *lacZ* reporter gene carrying *lexA* operator sites in the promoter. Sec24p (amino acids 34-926) interacted strongly with the central domain of Sec16p (amino acids 565-1235, Fig. 1 A). Sec24p also interacted strongly with Sec23p, as expected from the known association of these proteins in cell extracts (Fig. 1 A). No interaction was observed between Sec24p and the activation domain alone or between the central domain of Sec16p and Sec23p, indicating that the observed interactions are specific.

To define the region of Sec24p that interacts with Sec16p, we constructed fusions corresponding to the NH_2-terminal and COOH-terminal portions of Sec24p (Fig. 1). Both the NH_2-terminal and the COOH-terminal fusion showed a significant interaction with the central domain of Sec16p, but only the NH_2-terminal domain interacted with Sec23p (Fig. 1 A). These tests for interaction place the Sec23p binding site in the NH_2-terminal region of Sec24p, and suggests that both the NH_2-terminal and the COOH-terminal regions of Sec24p have sites that can bind to Sec16p.

We also used the two-hybrid system to identify the part of Sec16p that interacts with the COOH-terminal domain of Sec24p. The COOH-terminus of Sec24p (amino acids 666-926) fused to an acidic activation domain was tested for interaction with three Sec16p fragments fused to the *lexA* DNA-binding

domain (Fig. 1 B). The COOH-terminal domain of Sec24p interacted only with the central domain of Sec16p (Fig. 1 B). Sec23p, in contrast, interacted only with the COOH-terminal domain of Sec16p but not the central domain (Fig. 1 B). These results show that Sec23p and the COOH-terminal domain of Sec24p bind to different regions of Sec16p.

Sec24p Binds to the Central Domain of Sec16p in Yeast Extracts

To confirm the interactions detected by two-hybrid assay, we tested for the relevant protein-protein interactions in yeast extracts. Sec24p (amino acids 34-926), the NH_2-terminal domain, and the COOH-terminal domain of Sec24p were fused to glutathione-S-transferase (GST) and expressed in yeast from the *GAL1* promoter. To allow detection of the central domain of Sec16p (amino acids 565-1235), this domain was fused to invertase and similarly expressed from the *GAL1* promoter. GST-fusion and associated proteins were purified by affinity to glutathione beads from extracts prepared from yeast strains coexpressing one of the GST-fusion proteins and the Sec16p-invertase fusion. Binding to Sec16p was detected for GST fused to Sec24p, the Sec24p NH_2-terminus or the Sec24p COOH-terminus (Fig. 2 top panel, lanes 1-6) whereas no binding was detected for the control that used GST only (Fig. 2 top panel, lanes 7 and 8). Association between Sec16p and the two truncated Sec24p fusion proteins was reproducibly stronger than the association of the Sec16p and the Sec24p fusion that contained both the NH_2-terminal and COOH-terminal regions. The relatively strong binding of truncated fusion proteins could be because truncation of the protein gives better access to Sec16p binding sites.

The GST-Sec24p fusion proteins were also tested for their ability to bind to Sec23p expressed from the *GAL1*-promoter. Fusions to Sec24p or the

Sec24p NH_2-terminus, but not fusions to the Sec24p COOH-terminus or to GST alone, coprecipitated Sec23p (Fig. 2 bottom panel). These results are in complete agreement with the data obtained from the two-hybrid assays, and demonstrate that binding of Sec24p to Sec23p only requires the NH_2-terminus of Sec24p, while binding to the central domain of Sec16p is mediated by both the NH_2-terminus and the COOH-terminus of Sec24p.

Purification of Recombinant Sec23p and Sec24p from *E. coli*

In order to reconstitute the binding of Sec24p and Sec23p to Sec16p in vitro we required a source of pure Sec23p and Sec24p. Sec23p and Sec24p were expressed as GST-fusion proteins in *E. coli*, purified by their affinity to glutathione beads, and then released from the beads by cleavage with thrombin (see Materials and Methods). Sec23p purified in this manner was >90% pure as judged by Coomassie-stained SDS-PAGE (Fig. 3 A, lane 1). Optimal expression of Sec24p required induction at low temperature in a multiple protease deficient strain (*lon*$^-$ *ompT*$^-$) to prevent proteolytic degradation. After purification, the Sec24p preparation contained two major contaminating proteins (Fig. 3 A, lane 2); these proteins are probably bacterial proteins that bind non-specifically to glutathione beads, since they were also present - at much lower levels - in the Sec23p preparations, and did not react with anti-Sec24p antibodies. We used this partially purified preparation of Sec24p for all the experiments described below.

Bacterially expressed Sec24p and Sec23p were tested for their ability to bind to each other in vitro (Fig. 3 B). Beads containing purified GST-Sec23p coprecipitated all of the Sec24p added (Fig. 3 B, lanes 3 and 4), while no binding of Sec24p was detected to glutathione-beads alone (Fig. 3 B, lanes 5 and 6). Using the ability to bind to Sec23p as a criteria for function, all of the

recombinant Sec24p appeared to be active. The ability of recombinant Sec23p to bind to recombinant Sec24p in solution confirmed previous suggestions that no additional yeast proteins were required for association of Sec23p with Sec24p (Hicke *et al.*, 1992).

Binding of Sec23p and Sec24p to Sec16p In Vitro

To test binding of Sec23p and Sec24p to Sec16p in vitro, we purified the central and the COOH-terminal domain of Sec16p as GST-fusion proteins from yeast (see Materials and Methods). All purification steps were performed in the presence of 0.6 M NaCl to extract Sec16p fusion proteins from membranes. Under these extraction conditions, endogenous Sec23p and Sec24p did not copurify with Sec16p (note the absence of Sec23p and Sec24p in Fig. 4 A and B, lanes 3, 6, 9). No major contaminating proteins were present in the Sec16p preparations, as determined by Coomassie-stained SDS-PAGE; however, each of the Sec16p preparations contained a different set of proteins with faster mobility than the full length fusion proteins (Fig. 4 E). Some of these minor contaminants are likely degradation products of the Sec16p fusions, since a similar spectrum of bands was also detected upon long exposure of Western blots probed with anti-GST antibody (Fig. 4 A and B, and our unpublished data).

To test binding, Sec23p or Sec24p (2 pmol each) were added to the purified GST-Sec16p fusion proteins (11-13 pmol) bound to glutathione beads under conditions of low salt (45 mM NaCl). After incubation, the beads were collected and washed by centrifugation and bound proteins ("B" in Fig. 4 A and B) were compared to unbound proteins present in the supernatant ("S" in Fig. 4 A and B). A large fraction of Sec24p bound to the central domain of Sec16p (Fig. 4 A, lane 1) and a smaller, but still significant amount bound to the COOH-terminal domain of Sec16p (Fig. 4 A, lane 4). In the control, Sec24p did not bind to GST alone (Fig. 4 A, lane 7).

As expected, Sec23p behaved differently from Sec24p in this assay. Sec23p bound efficiently to the COOH-terminus of Sec16p (Fig. 4 B, lane 4), but no Sec23p bound to the central domain of Sec16p or to GST alone (Fig. 4 B, lanes 1 and 7). These data are consistent with the results of the two-hybrid and coprecipitation experiments and clearly demonstrate that the COPII coat components Sec23p and Sec24p bind to two distinct regions of Sec16p.

Sec23p and Sec24p copurify as a 300-400 kD complex from yeast cytosol (Hicke *et al.*, 1992). To test whether Sec23p and Sec24p can form a complex when bound to Sec16p, we added both Sec23p and Sec24p to GST fused to the central domain of Sec16p. If Sec24p can bind simultaneously to the central domain of Sec16p and to Sec23p, Sec24p should recruit Sec23p to the GST-Sec16p fusion protein. Importantly, these experiments employed a Sec16p fusion to which Sec23p alone would not bind. In the presence of Sec24p, a significant portion of Sec23p bound to the central domain of Sec16p (Fig. 4 C, lanes 3 and 4, compare to Fig. 4 B, lanes 1 and 2). Furthermore, the presence of Sec23p significantly increased the amount of Sec24p bound to the central domain of Sec16p (Fig. 4 C, compare lanes 1 and 2 and lanes 3 and 4). Optimal binding of Sec24p to the central domain of Sec16p required stoichiometric amounts of Sec23p (Fig. 4 D). These data argue that Sec24p has distinct binding sites for the central domain of Sec16p and for Sec23p, and show that Sec23p and Sec24p can bind to Sec16p as a complex.

To evaluate the relative strengths of these protein-protein interactions, we examined the stability of binding at different concentrations of NaCl (Fig. 5). Binding of Sec24p to the central domain of Sec16p was disrupted by 150 mM NaCl (Fig. 5 A), while binding of Sec23p to the COOH-terminal of Sec16p was stable up to 300 mM NaCl (Fig. 5 C). The presence of Sec23p did not affect the salt sensitivity of the interaction between Sec24p and the central domain of

Sec16p (Fig. 5 B). These data suggest that Sec23p binds more tightly to Sec16p than Sec24p, and argue that the stability of the interaction between Sec24p and the central domain of Sec16p is unchanged by Sec23p.

Discussion

Previous studies have shown that Sec23p, Sec24p and Sec16p are constituents of the protein coat of vesicles that bud from the ER (Barlowe *et al.*, 1994 and Espenshade *et al.*, 1995). Sec23p binds to Sec24p in a complex of 300-400 kD that can be isolated from the cytosol (Hicke *et al.*, 1992), and Sec23p binds to the COOH-terminal region of Sec16p (Espenshade *et al.*, 1995). In this paper we show that Sec24p also binds to Sec16p. In tests of truncated Sec16p molecules for binding, Sec24p was found to bind to a central region of Sec16p and, more weakly, to the COOH-terminal region of Sec16p, whereas Sec23p only bound to the COOH-terminal region of Sec16p. These binding interactions were reconstituted in vitro using recombinant Sec23p and Sec24p expressed in *E. coli* and GST-Sec16p fusion proteins purified from yeast. Importantly, in vitro binding of Sec24p to Sec16p did not require the presence of Sec23p, and binding of Sec23p to Sec16p did not require Sec24p.

Since Sec24p bound to the central domain of Sec16p could also recruit Sec23p to bind to this region of Sec16p, we suggest that Sec23p and Sec24p can bind to Sec16p in a ternary complex. It is possible that Sec23p and Sec24p are always in a complex and that the proteins bind to Sec16p as a unit. Alternatively, Sec23p and Sec24p may bind to Sec16p in sequential steps of coat assembly to build a Sec23p/Sec24p/Sec16p complex. Interestingly, Sec23p appears to positively regulate the interaction between Sec24p and Sec16p since the presence of Sec23p increases the amount of Sec24p that will bind to Sec16p in vitro. This increased binding could simply reflect the stoichiometry of Sec24p binding to Sec16p. For example, if the Sec23p/Sec24p complex contains two copies of Sec24p, formation of a Sec23p/Sec24p complex should double the number Sec24p molecules that can bind to each molecule of Sec16p. Alternatively, increased binding could

result from a conformational change in Sec24p induced by Sec23p that increases the affinity of Sec24p for Sec16p. The Sec23p/Sec24p/Sec16p complex showed similar sensitivity to 150 mM salt as the Sec24p/Sec16p complex suggesting that Sec23p does not greatly increase the strength of the interaction between Sec24p and Sec16p.

Because Sec16p, Sec23p and Sec24p are all components of the COPII vesicle coat, the protein-protein interactions that we have detected are likely to reflect subunit contacts that form during coat assembly on the ER membrane and that exist in the completed vesicle coat. By testing subdomains of Sec16p and Sec24p for interactions we have succeeded in identifying the region in these two molecules that are responsible for particular protein-protein interactions. From these interactions, a view of the relative arrangement of Sec16p, Sec23p and Sec24p proteins has begun to emerge (Fig. 6). Sec23p and Sec24p appear to lie next to each other along the Sec16p molecule: Sec23p binds to the COOH-terminal region of Sec16p and Sec24p binds to both the central domain of Sec16p and to the COOH-terminal domain of Sec16p. Given that the COOH-terminal region of Sec24p contacts the central region of Sec16p and that the NH_2-terminal region of Sec24p contacts Sec23p the most likely arrangement of Sec24p is with its NH_2-terminus oriented towards the COOH-terminus of Sec16p. The complex that we propose - which includes Sec23p, Sec24p and Sec16p - has not been isolated intact from yeast cells presumably because the high salt extraction conditions that are required to liberate Sec16p from the membrane also disrupt binding of Sec23p and Sec24p to Sec16p.

The ability of Sec23p and now Sec24p to bind to Sec16p reinforces the proposal that Sec16p acts as a membrane receptor to recruit soluble coat proteins to the membrane. The arrangement of cytosolic coat components along

Sec16p suggests a pathway for how COPII coat assembly on Sec16p takes place. Sar1p is currently the best candidate for the regulatory molecule that controls the initiation of vesicle coat assembly. Two proteins that we have identified to bind to the COOH-terminus of Sec16p are Sec23p and Sed4p (Espenshade *et al.*, 1995; Gimeno *et al.*, 1995). Sed4p genetically interacts with Sar1p (Gimeno *et al.*, 1995), and Sec23p is a GTPase activating protein for Sar1p (Yoshihisa *et al.*, 1993). An attractive possibility is that Sar1p initiates the coat assembly process by regulating assembly events that occur at the COOH-terminus of Sec16p. One of the early assembly steps - possibly mediated by Sar1p in its GTP-bound form - could be the recruitment of Sec23p to bind to the COOH-terminus of Sec16p. Binding of Sec23p to Sec16p could, in turn, recruit Sec24p to bind to the central domain of Sec16p. An attractive feature of this model is that it explains how an initiating event at the COOH-terminus of Sec16p could lead to the regulated, sequential assembly of a large multisubunit complex on the membrane. The availability of pure proteins, both coat components and the template onto which they assemble, will allow us to test this hypothesis.

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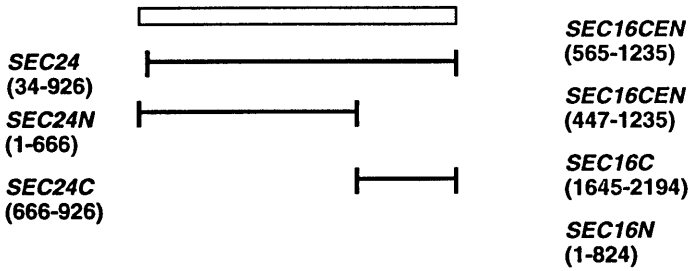
Figure 1. Two-hybrid interactions between Sec16p, Sec24p and Sec23p. The extent of each of the gene fragments of *SEC24* and *SEC16* are indicated by gene diagrams and by the amino acids numbers included in each construct given in brackets. Each interaction was evaluated by β-galactosidase assays of two to four independent transformants for which the mean activity is given (the standard deviation was always less than 20%).

A

LexA-DNA Binding Domain Fused to	Activation Domain fused to: *SEC16CEN* (565-1235)	*SEC23* (1-768)	no fusion
	β-galactosidase activity (U)		
SEC24 **(34-926)**	**3508**	**11924**	54
SEC24N **(1-666)**	**373**	**3632**	< 20
SEC24C **(666-926)**	**1138**	62	69
SEC23 **(1-768)**	< 20	66	33



B

LexA-DNA Binding Domain Fused to	Activation Domain fused to: *SEC24C* (666-926)	*SEC23* (1-768)	no fusion
	β-galactosidase activity (U)		
SEC16N **(1-824)**	< 20	< 20	< 20
SEC16CEN **(447-1235)**	**385**	42	< 20
SEC16C **(1645-2194)**	< 20	**3651**	< 20

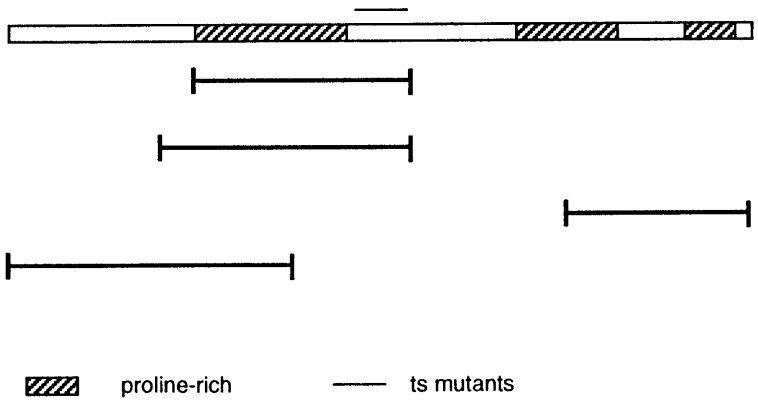


Figure 2. Affinity isolation of complexes between Sec24p, Sec16p and Sec23p from yeast extracts. The indicated fragments of Sec24p fused to GST (lanes 1-6) or GST only (lanes 7 and 8) were coexpressed with either the central domain of Sec16p fused to invertase (pRH317, upper panel) or Sec23p (pPE123, lower panel). GST-fusion and associated proteins were purified by affinity to glutathione-agarose. Proteins were detected by Western blotting after SDS-PAGE on a 6% gel. T = total, B = bound. Lanes 1, 3, 5, 7: 100,000 x *g* supernatant from 0.044 OD_{600} U of cells. Lanes 2, 4, 6, 8: material bound to beads from 0.73 OD_{600} U of cells.

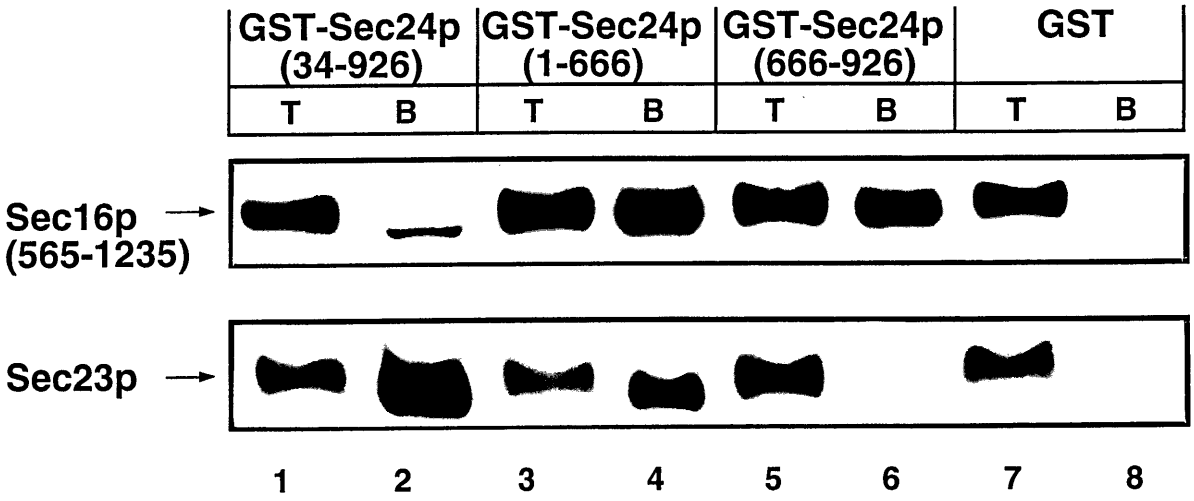
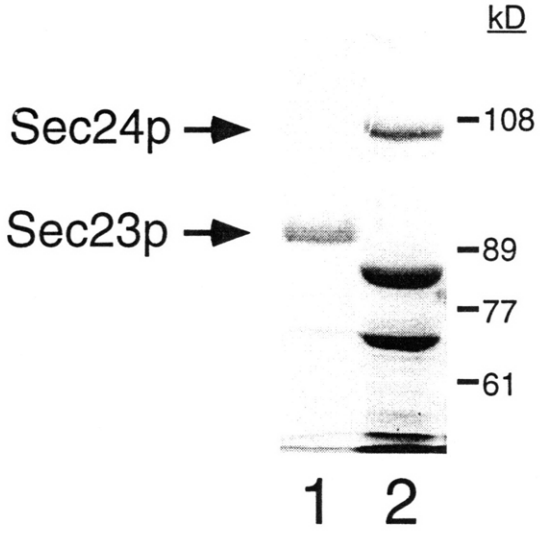

GST-Sec24p (34-926)
GST-Sec24p (1-666)
GST-Sec24p (666-926)
GST
T
B
T
B
T
B
T
B
Sec16p (565-1235)
Sec23p
1
2
3
4
5
6
7
8

Figure 3. Sec23p and Sec24p expressed in *E. coli* bind to each other. (A) Purified *E. coli* recombinant Sec23p (7 pmol, lane 1) and Sec24p (8 pmol, lane 2) were resolved by SDS-PAGE on a 6% gel and stained by Coomassie brilliant blue. (B) Immobilized *E. coli* recombinant GST-Sec23p (11 pmol; lanes 1-4) or glutathione beads only (lanes 5-6) were incubated with 6 pmol of *E. coli* recombinant Sec24p or buffer for 1 h at 22°C. Bound (B) and soluble (S) proteins were separated by SDS-PAGE on a 6% gel and stained with Coomassie brilliant blue.

A


kD
Sec24p
Sec23p
108
89
77
61
1 2

B

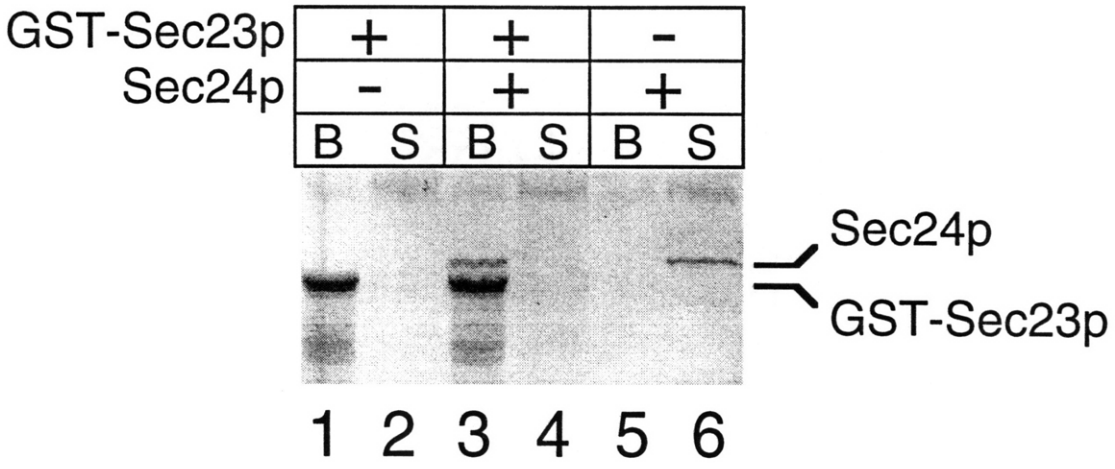
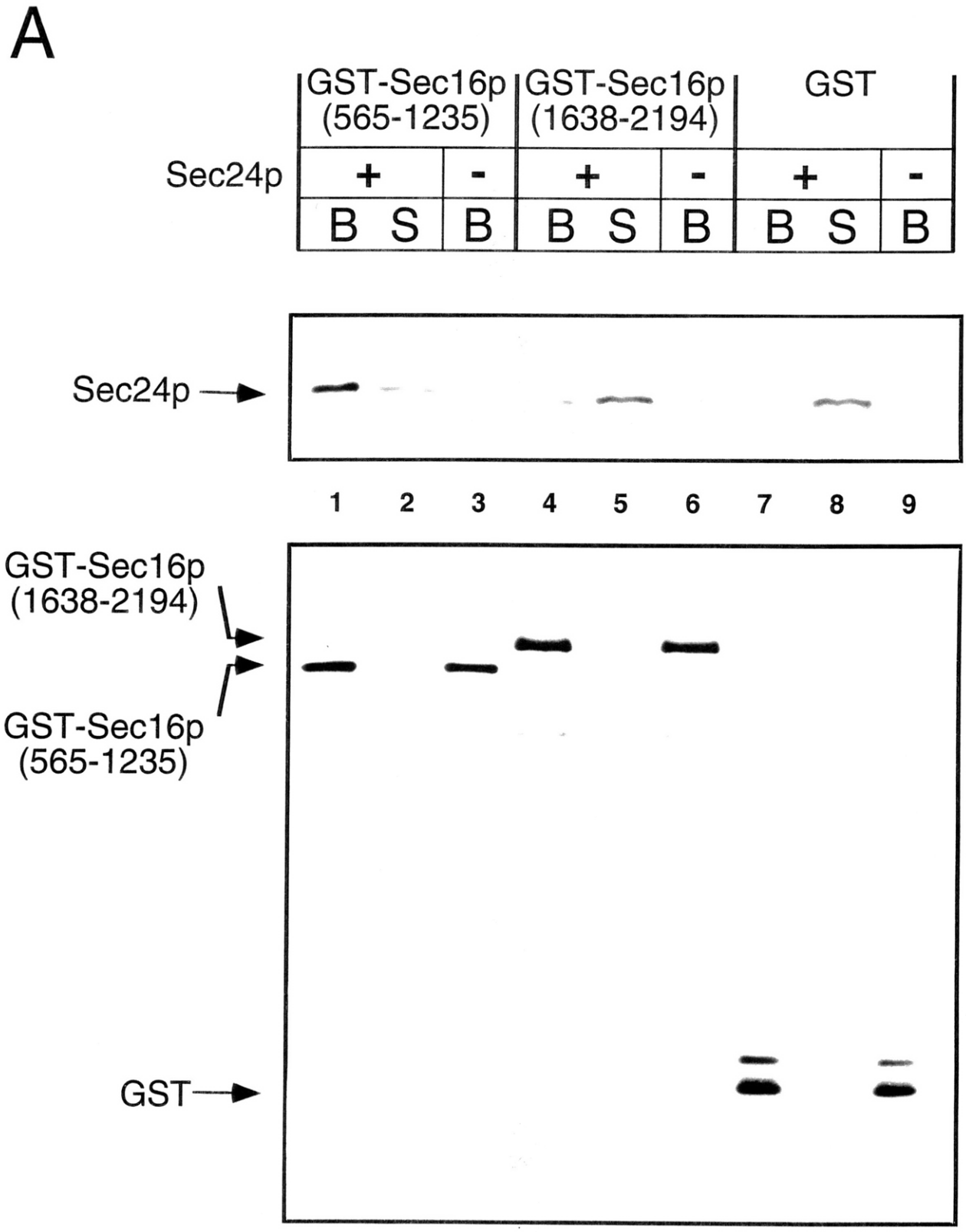
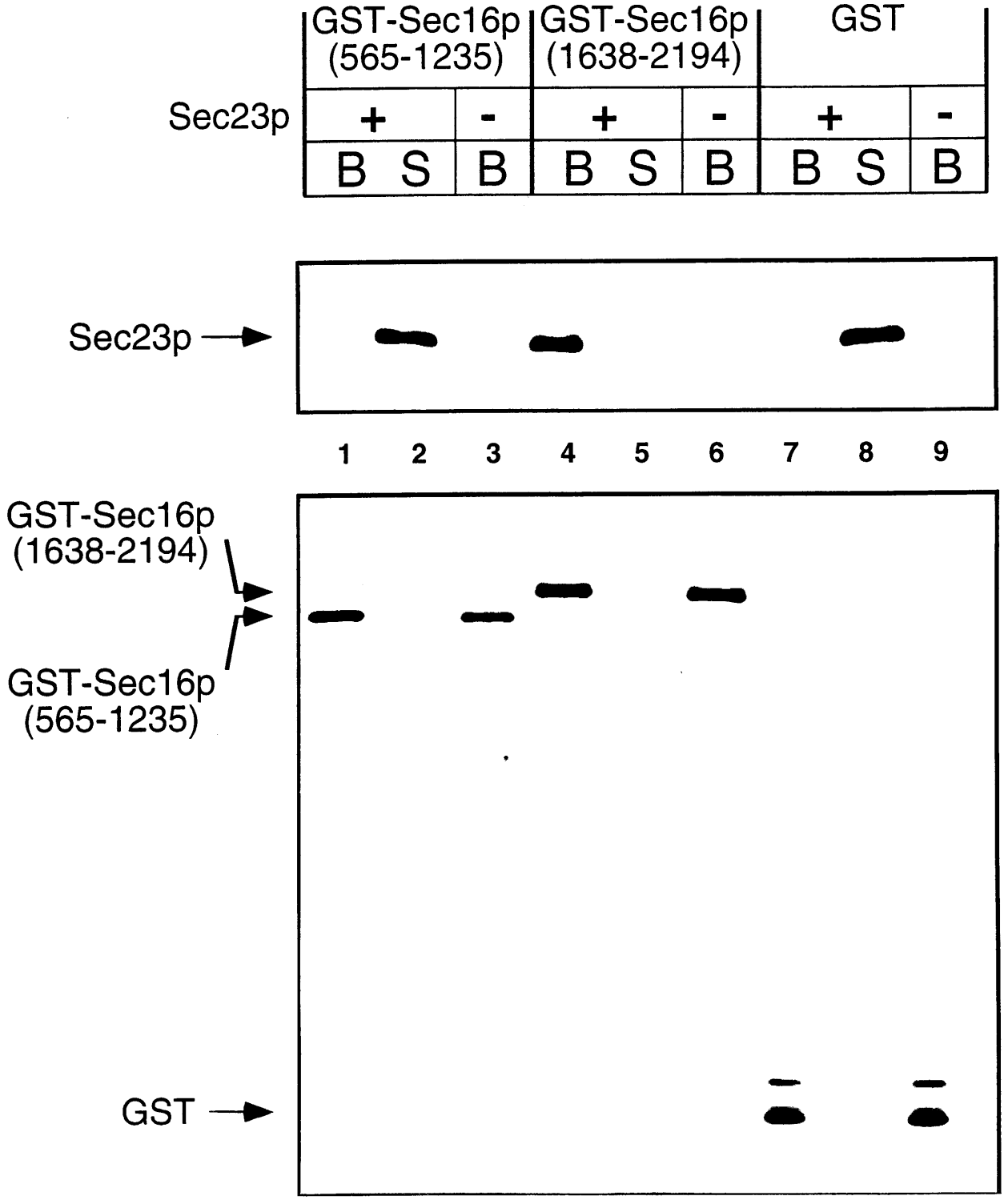

GST-Sec23p + + -
Sec24p - + +
B S B S B S
Sec24p
GST-Sec23p
1 2 3 4 5 6

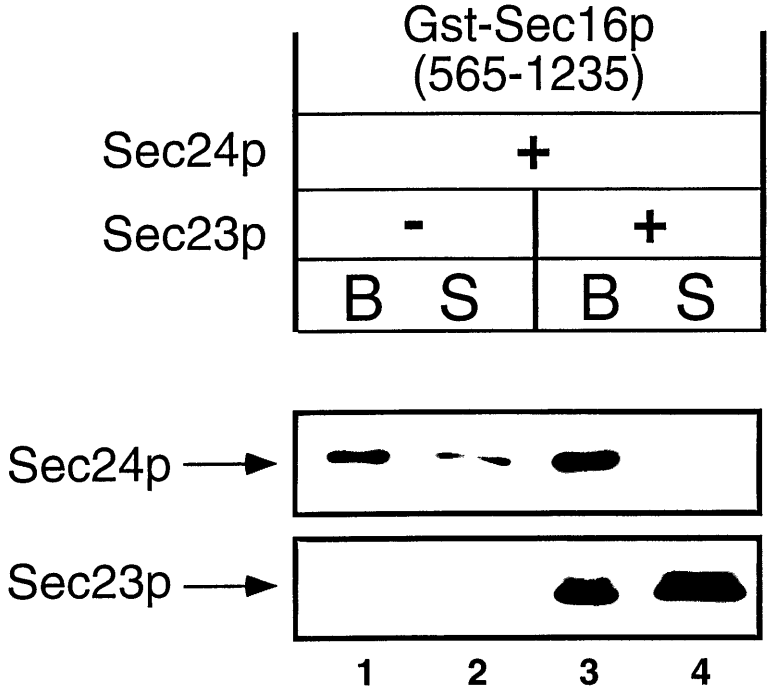
Figure 4. Sec24p binds to the central domain of Sec16p in vitro. The binding reactions in parts (A) and (B) contain one of the following GST-fusion proteins immobilized on glutathione agarose: lanes 1-3, 11 pmol of GST-Sec16p$_{(565-1235)}$; lanes 4-6, 13 pmol of GST-Sec16p$_{(1638-2194)}$; lanes 7-9, 11 pmol of GST only. To test binding, the immobilized proteins were incubated with 2 pmol of recombinant Sec24p (A), 2 pmol of recombinant Sec23p (B), or buffer for 1 h at 22°C. Bound (B) and soluble (S) fractions were resolved by SDS-PAGE on 10% gels and proteins were detected by Western blotting. (C) GST-Sec16p$_{(565-1235)}$ (11 pmol) was incubated with 2 pmol of recombinant Sec24p (lanes 1-4) and either 3 pmol recombinant Sec23p (lanes 3 and 4) or buffer (lanes 1 and 2) for 1 h at 22°C. Bound (B) and soluble (S) fractions were recovered as above. Proteins were detected by Western blotting after SDS-PAGE on a 10% gel. (D) GST-Sec16p$_{(565-1235)}$ (11 pmol) was incubated with 2 pmol of recombinant Sec24p (lanes 1-4) and the indicated amounts of recombinant Sec23p for 1 h at 22°C. Bound (B) and soluble (S) fractions were recovered as above. Sec24p was detected by Western blotting after SDS-PAGE on a 8% gel. Blots were quantitated by densitometry and the average from two independent experiments is shown. % Bound = B / B + S . (E) The GST-fusion proteins affinity-purified from yeast that were used in the binding reactions were separated by SDS-PAGE on a 10% gel and stained with Coomassie brilliant blue: lane 1, 17 pmol of GST-Sec16p$_{(565-1235)}$; lane 2, 20 pmol of GST-Sec16p$_{(1638-2194)}$; lane 3, 17 pmol of GST.


A
GST-Sec16p (565-1235)
GST-Sec16p (1638-2194)
GST
Sec24p
+
-
+
-
+
-
B S B B S B B S B
Sec24p
1 2 3 4 5 6 7 8 9
GST-Sec16p (1638-2194)
GST-Sec16p (565-1235)
GST

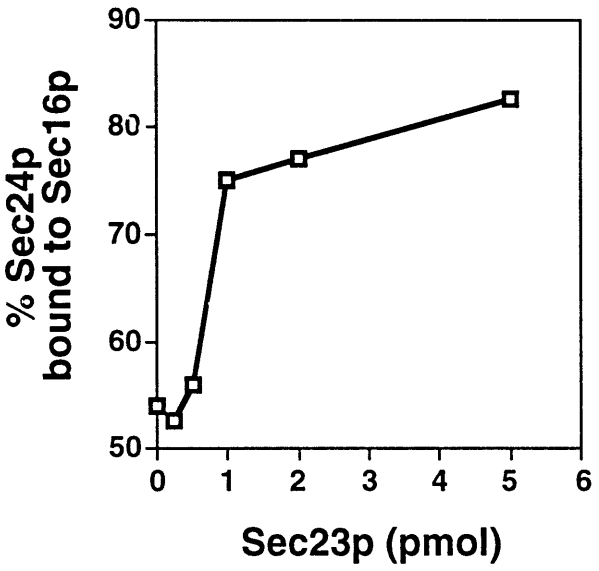
B


GST-Sec16p (565-1235)
GST-Sec16p (1638-2194)
GST
Sec23p
+
-
+
-
+
-
B S B B S B B S B
Sec23p
1 2 3 4 5 6 7 8 9
GST-Sec16p (1638-2194)
GST-Sec16p (565-1235)
GST

C



D



E

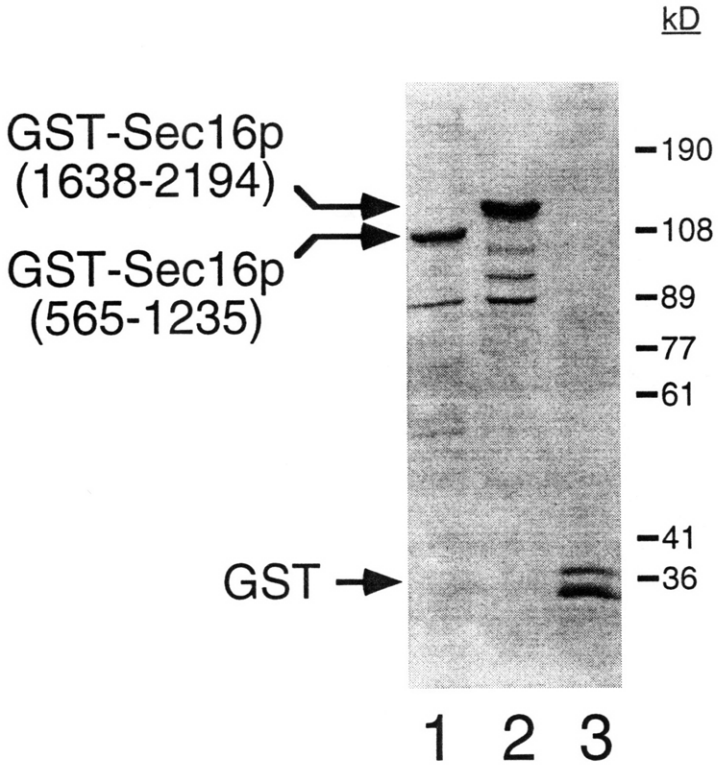

kD
GST-Sec16p
(1638-2194)
GST-Sec16p
(565-1235)
GST
190
108
89
77
61
41
36
1 2 3

Figure 5. Salt sensitivity of the interactions between Sec23p or Sec24p and Sec16p. Binding was performed in the presence of the indicated amounts of NaCl as described in Figure 4. Proteins were detected by Western blotting after SDS-PAGE on a 8% gel and quantitated by densitometry. The average of two independent experiments is shown. % Bound = B / B + S . (A) Binding of Sec24p to the central domain of $Sec16p_{(565-1235)}$. (B) Binding of Sec24p to the central domain of $Sec16p_{(565-1235)}$ in the presence of 2 pmol Sec23p. (C) Binding of Sec23p to the COOH-terminal domain of $Sec16p_{(1638-2194)}$.

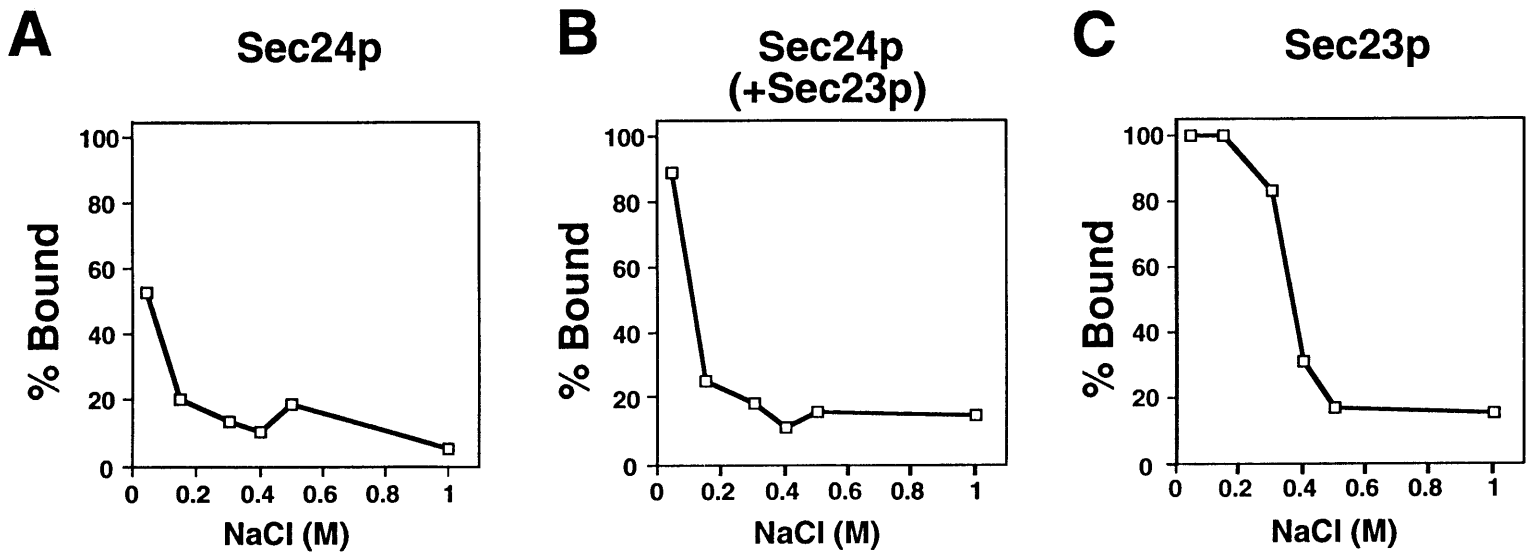

A
Sec24p
% Bound
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
NaCl (M)
B
Sec24p
(+Sec23p)
% Bound
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
NaCl (M)
C
Sec23p
% Bound
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
NaCl (M)

Figure 6. Proposed interactions between cytosolic COPII coat components and Sec16p.

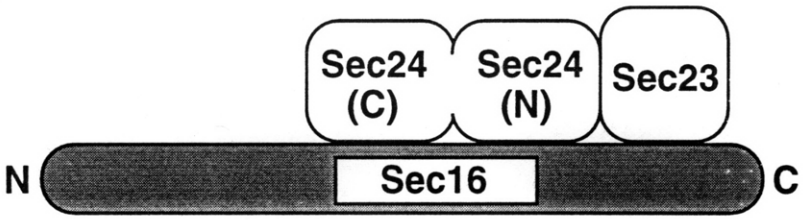

Sec24
(C)
Sec24
(N)
Sec23
N
Sec16
C

Table I. Plasmids

Plasmid	Description	Source or Reference
pRS315	centromere vector marked with *LEU2*	Sikorski and Hieter, 1989
pRS316	centromere vector marked with *URA3*	Sikorski and Hieter, 1989
pCT3	centromere vector marked with *URA3*	Thompson et al., 1993
pAF70	*SEC24* in pCT3	This study
pEG202	*lexA* DNA binding domain in a 2μ vector marked with *HIS3*	Gyuris et al., 1993
pGILDA	*lexA* DNA binding domain in a 2μ vector marked with *HIS3*	Shaywitz and Kaiser, unpublished
pJG4-5	acidic activation domain in a 2μ vector marked with *TRP1*	Gyuris et al., 1993
pGAD-GH	*GAL4* activation domain in a *CEN* vector marked with *LEU2*	Hannon et al., 1993
pSH18-34	*lacz* gene under control of 8 *lexA* DNA binding sites in a 2μ vector marked with *URA3*	Gyuris et al., 1993
pRH286	*SEC24* (34-926) in pEG202	This study
pRH321	*SEC24* (1-666) in pEG202	This study
pRH347	*SEC24* (666-926) in pEG202	This study
pDS79	*SEC23* in pGILDA	This study
pPE58	*SEC16* (1645-2194) in pEG202	Espenshade et al., 1995
pPE59	*SEC16* (1-824) in pEG202	Espenshade et al., 1995
pPE62	*SEC16* (447-1235) in pEG202	This study
pPE81	*SEC23* in pJG4-5	Espenshade et al., 1995
pPE167	*SEC16* (565-1235) in pGAD-GH	This study
pRH346	*SEC24* (666-926) in pJG4-5	This study
pRD56	*GAL1* promoted *GST* in pRS316	Espenshade et al., 1995
pRH305	*GAL1* promoted *GST-SEC24* (34-926) in pRS316	This study
pRH348	*GAL1* promoted *GST-SEC24* (1-666) in pRS316	This study
pRH360	*GAL1* promoted *GST-SEC24* (666-926) in pRS316	This study
pRH317	*GAL1* promoted *SEC16* (565-1235)-*SUC2* in pRS315	This study
pPE123	*GAL1* promoted *SEC23* in pRS315	This study
pRH323	*GAL1* promoted *GST-SEC16* (565-1235) in pRS316	This study
pPE122	*GAL1* promoted *GST-SEC16* (1638-2194) in pRS316	Gimeno et al., 1995
pGEX-2T	bacterial *GST* expression vector	Smith, 1993
pPE124	*SEC23* in pGEX-2T	This study
pPE166	*SEC24* (1-926) in pGEX-2T	This study

Note: The numbers in parenthesis indicate the amino acid numbers of the preceding gene's product

Chapter IV

Role of Sec16p in the Regulation of Vesicle Formation

Preface

The experiments involving the BIAcore biosensor were performed in collaboration with Chris Williams at Millenium Pharmaceuticals Inc., Cambridge, MA.

Abstract

The formation of COPII coated ER to Golgi transport vesicles requires the functions of seven genes - *SEC12*, *SEC23*, *SEC24*, *SEC13*, *SEC31*, *SEC16*, and *SAR1* (Schekman and Orci, 1996). Our studies focus on the role of Sec16p in this process. Genetic and biochemical evidence suggest that Sec16p functions as an organizer on the ER membrane for the assembly of the soluble COPII complexes - Sec13p/Sec31p, Sec23p/Sec24p, and Sar1p - that along with Sec16p eventually become components of the vesicle coat (Espenshade *et al.*, 1995; Gimeno *et al.*, 1996; Shaywitz *et al.*, 1997). Consistent with this model is the fact that Sec16p binds to Sec23p, Sec24p and Sec31p. In this report, we further investigate these binding interactions. We determine the dissociation constant for binding of Sec23p to Sec16p to be 1.3×10^{-8} M. Using in vitro binding studies, we demonstrate that Sec12p binds directly to the COOH-terminus of Sec16p. In addition, Sec23p and Sec12p compete for binding to the COOH-terminus of Sec16p. These data indicate that Sec16p may play a pivotal role not only in the assembly of the COPII coat, but also in the regulation of vesicle formation.

Introduction

Formation of COPII coated ER to Golgi transport vesicles is controlled by the action of the small GTP-binding protein, Sar1p (Schekman and Orci, 1996). Sar1p is recruited to the ER membrane through association with Sec12p, an ER resident protein that can function as a guanine nucleotide exchange factor for Sar1p (Fig. 1, Step 1). Sec12p activates Sar1p by catalyzing exchange of GTP for GDP (Barlowe and Schekman, 1993). Sec23p, which functions as a GTPase activating protein (GAP) for Sar1p, is then believed to be recruited to the membrane by activated Sar1p-GTP (Yoshihisa *et al.*, 1993; Schekman and Orci, 1996).

Both genetic and biochemical evidence suggest that Sec16p, a protein required for vesicle formation in vivo, functions to organize the assembly of Sec23p and the other soluble COPII proteins on the ER membrane. In one model, Sec23p/Sec24p bind directly to the ER peripheral membrane protein Sec16p in what is the first step in vesicle coat assembly (Fig. 1, Step 2) (Espenshade *et al.*, 1995; Gimeno *et al.*, 1996). Recent experiments demonstrated that in addition to binding to Sec13p, Sec31p can bind to Sec16p, Sec23p, and Sec24p (Shaywitz *et al.*, 1997). Sec13p/Sec31p is likely recruited to the membrane through the interaction of Sec31p with these other COPII proteins (Fig. 1, Step 3). Polymerization of COPII subunits leads to vesicle budding and the creation of a vesicle that lacks Sec12p, but has a coat composed of the COPII proteins: Sec16p, Sec23p/Sec24p, Sec13p/Sec31p, and Sar1p (Espenshade *et al.*, 1995; Schekman and Orci, 1996). Stability of the vesicle coat is controlled by the nucleotide bound state of Sar1p (Oka and Nakano, 1994; Barlowe *et al.*, 1994). Hydrolysis of GTP by Sar1p, stimulated by Sec23p, causes the coat to disassemble and allows fusion of the vesicle with the Golgi apparatus.

Sec16p appears to be functionally positioned at the center of the COPII coat assemblage. Consequently, Sec16p is in an ideal position to participate in the regulation of vesicle formation. In this study, we demonstrate that Sec16p also binds to Sec12p and that Sec23p competes for this binding to Sec16p. Control of Sec12p and Sec23p binding to Sec16p by the small GTP-binding protein Sar1p may represent a key regulatory step in vesicle formation that would couple recruitment of Sar1p to the membrane with the assembly of the COPII subunits on Sec16p.

Materials and Methods

General Techniques

Table I 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 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 procedures used Vent polymerase according to the manufacturer's specifications (New England Biolabs, Beverly, MA). Western blotting was performed as described (Espenshade *et al.*, 1995) with the following dilutions of antibodies: anti-Sec23p (1:1,000) (the kind gift of R. Schekman, University of California, Berkeley, CA) or anti-Sec12p [amino acids (aa) 1-354] (1:1,000) and anti-rabbit IgG, peroxidase coupled whole antibody from donkey (1:10,000, Amersham Corp., Arlington Heights, IL). Western blots were developed using chemiluminescence (Pierce, Rockford, IL). Protein concentrations were determined by the Bio-rad Protein Assay (Bio-rad Laboratories, Hercules, CA) using bovine serum albumin (Boehringer Mannheim, Indianapolis, IN) as a standard.

Construction of *sar1* Mutants

A plasmid encoding *SAR1* expressed from the *GAL1* promoter, pPE239, was created by inserting a BamHI/XbaI fragment from a derivative of pTY20 (Barlowe *et al.*, 1994) into pRS315. Point mutant variants of *SAR1*, pPE275 and pPE276, were constructed using the technique of splice overlap extension PCR (Taylor, 1991) and were cloned into pCD43 by digestion of the PCR fragment with BamHI/XbaI. The point mutations, *sar1T37N* and *sar1H77G*, were made using pPE239 as a DNA template, vector primers, and the following mutagenic

oligonucleotides: 5'-TGTAGCAATGTGTTCTTACCGGCAT-3', 5'-ATGCCGGTA-AGAACACATTGCTACA-3' for *sar1T37N* and 5'-CGAGCTTGAATACCACCAC-CCAAATC-3', 5'-GATTTGGGTGGTGGTATTCAAGCTCG-3' for *sar1H77G*. *SEC12* overexpression was directed from the *GAL10* promoter using the plasmid pPE262, a derivative of pRH152 (Gimeno *et al.*, 1995). Experiments analyzing the overexpression of the *sar1* mutants were performed using the yeast strain CKY473 (*MATα leu2-3,112 ura3-52* Gal$^+$) transformed with different plasmid combinations.

Purification of Sec12p (aa 1-354)

Sec12p (aa 1-354) was expressed as a fusion to glutathione *S*-transferase in the *Escherichia coli* strain CKB175 (Gimeno *et al.*, 1996). To construct a *SEC12 (aa 1-354)* expression vector, a 1.1 kb PCR fragment produced using oligonucleotide primers (5'-CGGGATTCATGAAGTTCGTGAC-AGCTAG-3', 5'-CGGAATTCCTATTTAGAGATTTTTTGTTTCATTGAGG-3') and wild-type yeast genomic DNA as a template was digested with BamHI/EcoRI (underlined) and ligated into the expression vector pGEX-2T to yield pPE223 (Smith and Johnson, 1988). The fusion protein was purified using glutathione Sepharose (Pharmacia Biotech, Piscataway, NJ) and the GST moiety was removed by digestion with thrombin as described (Espenshade *et al.*, 1995).

Purification of Sec23p

Sec23p with six histidine residues added to the COOH-terminus was expressed as a fusion to glutathione *S*-transferase in the *Escherichia coli* strain CKB175 (Gimeno *et al.*, 1996). The GST-Sec23p-6xHis expression plasmid, pPE229, was constructed by inserting into pPE124 a SphI/HindIII digested PCR fragment made using pPE124 as a template and oligonucleotide primers (5'-

GACAAGTAAAGACTTGAAGG-3', 5'-CCGCTCGAGAAGCTTCTAGTGATGGTG-ATGGTGATGTGCCTGACCAGAGACGGC-3'). The GST-Sec23p-6xHis fusion protein was purified first by affinity to glutathione Sepharose (Pharmacia Biotech) and the GST moiety was removed by cleavage with thrombin (Espenshade *et al.*, 1995). The cleaved protein Sec23p-6xHis was then further purified using Ni-NTA resin (Qiagen Inc., Santa Clarita, CA) according to the manufacturer's instructions. Purified Sec23p-6xHis was used for the in vitro binding studies to Sec16p. Sec23p without a histidine tag was used in experiments to measure the kinetics of Sec23p binding to Sec16p with the BIAcore and was purified as described (Gimeno *et al.*, 1996).

Sec12p Antiserum

Sec12p polyclonal rabbit antiserum was elicited against the cytosolic NH_2-terminal domain of Sec12p (aa 1-354) expressed in *Escherichia coli*. Antiserum was generated to purified Sec12p (aa 1-354) (see above) using two rabbits 824 and 825 as described previously (Covance Inc., Denver, PA; Griff *et al.*, 1992).

Preparation of GST-Sec16p Proteins and In Vitro Binding Assay

The yeast GST-Sec16p (aa 1892-2194) fusion protein was expressed from the *GAL1* promoter on the plasmid pPE255. The plasmid pPE255 was constructed by digesting a PCR fragment generated using the oligonucleotide primers (5'-CGCGGATCCATGTACTCACCAAGTGTGG-3', 5'-CGGAATTCTATT-AGTGATGGTGATGGTGATGTTGTATGTTATCCATTA-3') and pPE10 (*SEC16-HA* under control of the *GAL1* promoter in pRS315) with BamHI/EcoRI and inserting this fragment into pRD56. Construction of GST-Sec16p (aa 1892-2194) also resulted in the insertion of an influenza HA epitope at the NH_2-

terminus of Sec16p and a six histidine tag at the COOH-terminus. The bacterial expression plasmid encoding GST-Sec16p (aa 1892-2095), pPE271, also contained an influenza HA epitope at the NH_2-terminus and was created by inserting a PCR fragment digested with BamHI/EcoRI into pGEX-2T (Smith and Johnson, 1988). The PCR fragment was made using the oligonucleotide primers (5'-CGCGGATCCATGTACTCACCAAGTGTGG-3', 5'-CGGAATTCTT-AACGTGGCTTTGTCTTTGG-3') and pPE10 (*SEC16-HA* under control of the *GAL1* promoter in pRS315) as a template. Bacterial GST was expressed using pGEX-2T.

Yeast GST-Sec16p (aa 1892-2194) was expressed in CKY474 (*MATα leu2-3,112 ura3-52 pep4::LEU2* Gal^+). Yeast lysate containing the fusion protein was prepared as previously described (Gimeno *et al.*, 1996). Bacterially expressed GST-Sec16p (aa 1892-2095) and GST were expressed in CKB175 and lysates were prepared as described (Johnson and Corcoran, 1994).

In vitro binding experiments were performed as described (Gimeno *et al.*, 1996) with the exception that the binding buffer contained 25 mM Hepes, pH 6.8, 0.1% Triton X-100, 1 mM MgOAc, 0.5 mM DTT, and 0.25 mg/ml BSA. Purified proteins, either Sec12p (aa 1-354) or Sec23p (in 25 mM Hepes, pH 6.8, 150 mM KOAc, 1 mM MgOAc, and 0.02% Triton X-100) were added to a reaction containing GST proteins bound to glutathione Sepharose beads, and the final salt concentration was adjusted to 45 mM KOAc. For competition experiments, Sec12p (aa 1-354) and Sec23p were added to the reaction as a single solution. The amount of protein bound to the beads was analyzed by Western blotting and quantitated using NIH Image Software (National Institutes of Health, Bethesda, MD).

Biosensor Analysis of Sec23p Binding to Sec16p

Standard binding experiments and kinetic analysis of Sec23p binding to GST-Sec16p (aa 1892-2095) were performed using a BIAcore 2000 (BIAcore, Inc.; Uppsula, Sweden). GST-Sec16p (aa 1892-2095) was purified from bacterial lysates using glutathione Sepharose (Pharmacia Biotech) as described (Johnson and Corcoran, 1994). Recombinant Sar1p and Sec23p were purified from *E. coli* as described (Barlowe *et al.*, 1994; Gimeno *et al.*, 1996). All proteins were exchanged into 25 mM Hepes, pH 6.8, 150 mM KOAc, 1 mM MgOAc, and 0.02% Triton X-100 using Slide-a-lyzer dialysis cassettes (Pierce). A BIAcore Sensorchip CM5 (research grade) with immobilized GST antibodies was prepared using a standard amine coupling reaction and the BIAcore GST Antibody Kit (BIAcore, Inc.). GST-Sec16p (aa 1892-2095) was bound to the chip surface prior to each binding sequence. All binding studies were performed at 22˚C with a flow rate of 10 µl/min and using 25 mM Hepes, pH 6.8, 150 mM KOAc, 1 mM MgOAc, and 0.02% Triton X-100 as the driving buffer. Bound proteins were removed after each binding cycle by treatment with 100 mM NaOH.

Kinetic measurements were performed by injection of the analyte for 120s followed by dissociation in buffer flow for 180s. Kinetic analysis was done using multiple surface densities. No significant binding of analyte to the blank chip or to the antibody-coated chip was observed. Kinetic data from two independent tests using four concentrations of Sec23p (2 µM, 1 µM, 0.5 µM, and 0.25 µM) were used to determine the kinetic parameters. Rate constants for the binding of Sec23p to Sec16p were calculated using the kinetics software package BIAevaluation 3.0 (BIAcore, Inc.).

Results

Mapping of the Sec23p Binding Site on Sec16p

To better understand the assembly of the COPII vesicle coat and to quantitate the stability of the interactions that constitute the coat, we examined the binding of Sec23p to Sec16p in more detail. Previous experiments demonstrated that Sec23p can bind directly to the COOH-terminus of Sec16p (aa 1638-2194) in vitro (Gimeno *et al.*, 1996). To map the Sec23p binding site more exactly, we tested binding of Sec23p to two deletions of Sec16p fused to glutathione S-transferase (GST). Recombinant GST-Sec16p fusion proteins were purified from cell extracts using glutathione Sepharose beads, and GST-Sec16p immobilized on beads was incubated with recombinant Sec23p. The amount of bound Sec23p was compared to unbound protein present in the supernatant. Sec23p bound to both fusion proteins, GST-Sec16p (aa 1892-2194) and GST-Sec16p (aa 1892-2095), but not to GST alone (Fig. 2). These data demonstrate that ~200 amino acids of Sec16p (aa 1892-2095) are sufficient for binding to Sec23p.

Biosensor Analysis of Sec23p Binding to Sec16p

To quantitate the affinity of the Sec16p/Sec23p binding interaction in vitro, we measured the kinetics of association and dissociation by a surface plasmon resonance based technique on a BIAcore. The kinetic parameters of Sec23p binding to GST-Sec16p (aa 1892-2095), captured by immobilized GST antibodies, were determined in two separate experiments using four different concentrations of Sec23p. The calculated rate constants for binding were k_{on} = 1.4×10^5 $M^{-1}s^{-1}$ and k_{off} = 1.8×10^{-3} s^{-1}. An apparent dissociation constant of 1.3×10^{-8} M was derived from the observed rate constants ($k_{off}/k_{on} = K_{diss}$).

Overexpression of Sec12p Suppresses the Toxicity of a *sar1* GDP-restricted mutant, but not GTP-restricted mutant

Sec12p is the third component of the Sar1p GTPase cycle, in addition to Sar1p and Sec23p. In vitro biochemical experiments have demonstrated that the NH_2-terminus of Sec12p (aa 1-354) can facilitate Sar1p nucleotide exchange and that Sar1p shows increased binding to membranes in cells overexpressing Sec12p (Barlowe and Schekman, 1993; d'Enfert *et al.*, 1991a; d'Enfert *et al.*, 1991b). These data have led to a model in which Sec12p recruits Sar1p to the ER membrane and catalyzes exchange of GDP for GTP, thereby initiating vesicle formation. The recent development of well-characterized GDP and GTP conformational mutants of Sar1p allowed us to test this model in vivo.

Studies in mammalian cell assays involving the two trans-dominant mutants Sar1T39N and Sar1H79G have revealed that these mutations restrict Sar1p to the GDP and GTP bound conformations, respectively (Kuge *et al.*, 1994; Aridor *et al.*, 1995). Sar1T39N has a reduced affinity for GTP and inhibits vesicle budding from the ER as expected for a GDP-restricted mutant (Kuge *et al.*, 1994). The activated GTP-restricted mutant, Sar1H79G, can recruit COPII proteins to membranes and blocks ER to Golgi transport in vitro (Aridor *et al.*, 1995). To test the effect of overexpression of these mutant proteins on yeast cell growth, we made the corresponding mutations in yeast *SAR1*, *sar1T37N* and *sar1H77G*, and placed the mutant genes under the control of the *GAL* promoter. Overexpression of *sar1T37N* in a wild-type yeast strain inhibited cell growth, most likely due to an inability to form COPII vesicles (Fig. 3a). Specifically, overexpression of the GDP-restricted form of Sar1p, *sar1T37N*, may be toxic because Sar1T37Np competes with the less abundant wild-type Sar1p for binding to the exchange factor Sec12p. If this is the case,

overexpression of the exchange factor might suppress the toxicity and restore growth. Indeed, overexpression of *SEC12* from the *GAL* promoter partially suppressed the toxicity of sar1T37N overexpression (Fig. 3a), providing in vivo evidence for the model that Sec12p binds to Sar1p and functions as an exchange factor.

Hydrolysis of GTP by Sar1p is required for uncoating of COPII vesicles and fusion with the Golgi, but is not required for vesicle formation (Barlowe *et al.*, 1994; Oka and Nakano, 1994). According to this model, overexpression of the GTP-restricted mutant should lead to the formation of COPII vesicles with a stable coat that cannot fuse with the Golgi. Consistent with this idea, overexpression of *sar1H77G* in a wild-type strain inhibited growth, and this toxicity of *sar1H77G* was not rescued by the overexpression of *SEC12* (Fig. 3b). In addition, overexpression of *SEC23*, the GTPase activating protein for Sar1p, did not rescue growth of either mutant (data not shown). These data suggest that the trans-dominant *sar1* mutants act in a similar manner in yeast as in mammalian cells and provide support for the model that Sec12p is the exchange factor that activates Sar1p in vivo.

Sec12p Binds to the COOH-Terminus of Sec16p

In addition to binding to the COPII proteins - Sec23p, Sec24p, and Sec31p - Sec16p also binds to Sed4p, a close homologue of Sec12p (Gimeno *et al.*, 1995). Sec12p and Sed4p are both type II ER integral membrane proteins that share 45% amino acid identity in the NH_2-terminal, cytosolic domains. The cytosolic domain of Sed4p can bind to the COOH-terminus of Sec16p, but binding of Sec12p to Sec16p has not been detected (Gimeno *et al.*, 1995). Data suggesting a role for Sar1p in the regulation of Sec23p binding to Sec16p prompted the re-examination of binding between Sec12p and the

COOH-terminus of Sec16p.

To test binding of Sec12p to Sec16p, a soluble, recombinant protein, Sec12p (aa 1-354), that consists of the entire cytosolic domain of Sec12p was purified from bacterial extracts and assayed for binding to GST-Sec16p fusion proteins in vitro. GST-Sec16p proteins, immobilized on glutathione Sepharose, were incubated with Sec12p (aa 1-354). The amount of Sec12p (aa 1-354) bound to GST-Sec16p was compared to protein in the supernatant by Western blot. Sec12p (aa 1-354) bound to both GST-Sec16p (aa 1892-2194) and to GST-Sec16p (aa 1892-2095), but very little Sec12p protein was found to be associated with GST alone (Fig. 4). These data show that the cytosolic domain of Sec12p (aa 1-354), that catalyzes nucleotide exchange on Sar1p, can bind to the COOH-terminus of Sec16p. Furthermore, Sec12p binds to the same 200 amino acids that are capable of binding to Sec23p, the GTPase activating protein for Sar1p.

In a prior experiment, Sed4p bound to Sec16p in yeast cell extracts, but binding of Sec12p to Sec16p was not detected (Gimeno *et al.*, 1995). In the current experiments, binding of Sec12p to Sec16p was observed as well as binding of Sed4p to Sec16p (data not shown). The difference in the results of the two experiments may lie in the source of the proteins. When prepared from *E. coli*, GST-Sec16p and Sec12p (aa 1-354) bound stably to one another. In contrast, binding of Sec12p to Sec16p was not detected in yeast cell extracts suggesting that an inhibitor, or negative regulator, of binding may be present in yeast lysates. Consistent with this idea, Sec12p (aa 1-354) appears to bind to bacterial GST-Sec16p (aa 1892-2095) more tightly than yeast GST-Sec16p (aa 1892-2194) (Fig. 4, lanes 1-4). One possible explanation is that Sec16p is modified differently in yeast than in bacteria. Alternatively, Sed4p may simply have a higher affinity for Sec16p than Sec12p. Binding studies comparing the

interaction of Sed4p and Sec12p with Sec16p may help to explain these somewhat conflicting results.

Sec12p and Sec23p Compete for Binding to Sec16p

To test whether the binding sites for Sec12p and Sec23p on Sec16p were identical, we examined the binding of each protein to Sec16p in the presence of increasing amounts of the other protein. GST-Sec16p (aa 1892-2194), bound to glutathione beads, was incubated with 0.2 μM recombinant Sec23p. The amount of Sec23p bound to beads under these conditions was determined by Western blotting and defined as 100%. Binding of Sec23p to Sec16p was then assayed in the presence of increasing concentrations of Sec12p (aa 1-354). The presence of Sec12p (aa 1-354) in the reaction inhibited binding of Sec23p to Sec16p (Fig. 5a). Addition of 7.5 μM Sec12p (aa 1-354) reduced binding of Sec23p to 11% of the level seen with no Sec12p (aa 1-354) added.

In a similar experiment, 0.2 μM Sec12p (aa 1-354) was incubated with GST-Sec16p (aa 1892-2194) and increasing concentrations of Sec23p. Addition of Sec23p to the in vitro binding reaction prevented binding of Sec12p (aa 1-354) to Sec16p (Fig. 5b). Inclusion of 7.8 μM Sec23p in the reaction reduced binding of Sec12p (aa 1-354) to 7% of the level observed with no Sec23p added. Thus, Sec12p (aa 1-354) and Sec23p compete for binding to Sec16p (aa 1892-2194). These data demonstrate that Sec12p and Sec23p likely bind to identical, or overlapping, sites on Sec16p. Alternatively, binding of Sec23p could cause an allosteric change in Sec16p that prevents binding of Sec12p, or vice versus.

Discussion

Both genetic and biochemical approaches have led to the identification of at least seven genes required for the formation of ER to Golgi transport vesicles (Schekman and Orci, 1996). However, we still lack a detailed understanding of how the proteins encoded by these genes cooperate at the molecular level to regulate and form a COPII-coated vesicle. The results of the experiments in this study suggest that Sec16p may play a central role in both the regulation of vesicle formation and the assembly of the COPII coat.

One unanswered question regarding the assembly of the COPII coat is how recruitment of Sec23p by Sar1p to the ER membrane is coupled to the binding of Sec23p to Sec16p (Fig. 1, Step 2). Results in this study provide clues as to how this may be coordinated. Previous experiments have shown that the cytosolic NH_2-terminal domain of Sed4p, which is 45% identical to Sec12p, binds to the COOH-terminus of Sec16p (aa 1638-2194) in yeast cell extracts (Gimeno *et al.*, 1995). Here, we demonstrate that the cytosolic NH_2-terminus of Sec12p that catalyzes nucleotide exchange on Sar1p can also bind to Sec16p (aa 1892-2095). Furthermore, we show that Sec12p (aa 1-354) and Sec23p compete for binding to Sec16p (aa 1892-2095). Binding of both the exchange factor, Sec12p, and the GTPase-activating protein, Sec23p, for Sar1p to the same site on Sec16p suggests an attractive model for the regulation of vesicle formation at the ER membrane.

Sec12p bound to Sec16p creates a high affinity binding site for Sar1p which is recruited to the ER membrane (Fig. 6). Sec12p activates Sar1p by exchange of GTP for GDP, and this results in the recruitment of Sec23p to the ER membrane. Sec23p binds to Sec16p and displaces Sec12p allowing it to diffuse away from the vesicle bud site. Binding of Sec23p results in the recruitment of the other COPII subunits and ultimately vesicle budding. Finally,

hydrolysis of GTP by Sar1p destabilizes the interaction of Sec23p and Sec16p resulting in the disassembly of the COPII coat, facilitating vesicle fusion with the Golgi apparatus.

Evidence for such a model is beginning to emerge. In this study, we show that Sec12p and Sec23p bind to competing sites on Sec16p, and preliminary data suggest that Sar1p-GMP-PNP may stimulate binding of Sec23p to Sec16p. In a recent paper that demonstrates a requirement for Sec16p in the in vitro vesicle budding assay, Campbell and Schekman (1997) also propose that Sec12p and Sec16p may combine to form a high affinity binding site for Sar1p. This suggestion stems from in vitro data that implicate Sec16p in the concentration of Sar1p at the site of vesicle formation. Binding studies that examine the effect of Sar1p on the competition of Sec12p and Sec23p for binding to Sec16p will help to test the validity of these hypotheses.

Clearly, questions remain unanswered about the mechanism of ER to Golgi transport vesicle formation. With many of the proteins required for this process identified, the task is now to decipher the molecular interactions that generate a COPII coated vesicle. The development of purified, soluble Sec16p will be critical to this effort. The data presented here support a model that serves as a framework for future experimental design.

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Figure 1. Model for the assembly of COPII subunits during vesicle formation. *Step 1*, Sec12p recruits Sar1p to the ER membrane and exchanges GTP for GDP. *Step 2*, activated Sar1p-GTP recruits Sec23p/Sec24p to bind to Sec16p. *Step 3*, Sec13p/Sec31p binds to Sec16p and Sec23p/Sec24p. Adapted from Shaywitz *et al.*, 1997.

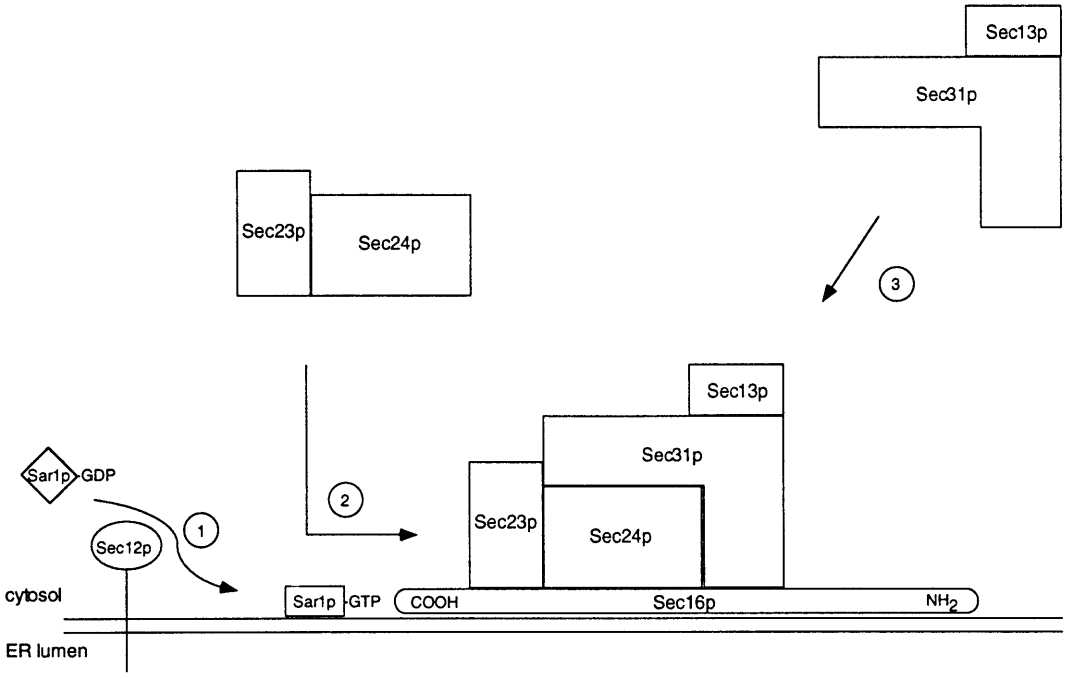

Sec13p
Sec31p
Sec23p
Sec24p
3
Sec13p
Sec31p
Sar1p
GDP
2
Sec23p
Sec24p
1
Sec12p
cytosol
Sar1p
GTP
COOH
Sec16p
NH2
ER lumen

Figure 2. Mapping of the Sec23p binding domain. Binding reactions contained one of the following GST fusion proteins immobilized on glutathione Sepharose beads: lanes 1 and 2, 60 pmol of yeast GST-Sec16p (aa 1892-2194); lanes 3 and 4, 15 pmol of bacterial GST-Sec16p (aa 1892-2095); lanes 5 and 6, 15 pmol of bacterial GST. To assess binding, we incubated 1 pmol of recombinant Sec23p with the immobilized proteins for 1 hr at 22°C. One-tenth of the bound (B) and soluble (S) fractions were resolved by SDS-PAGE and Sec23p was detected by Western blotting with Sec23p antiserum.

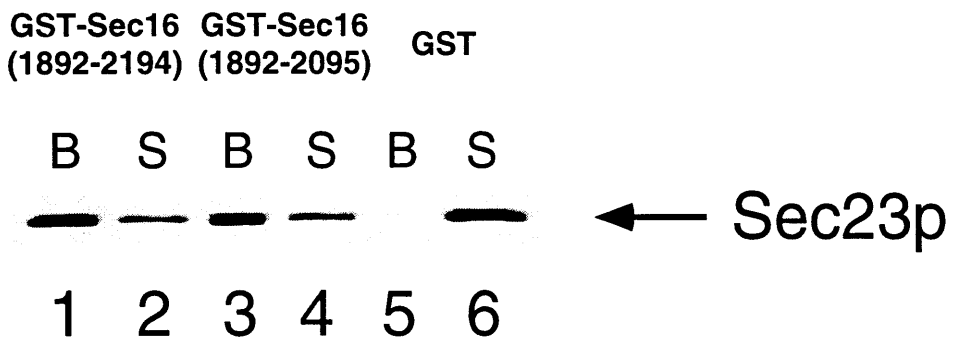
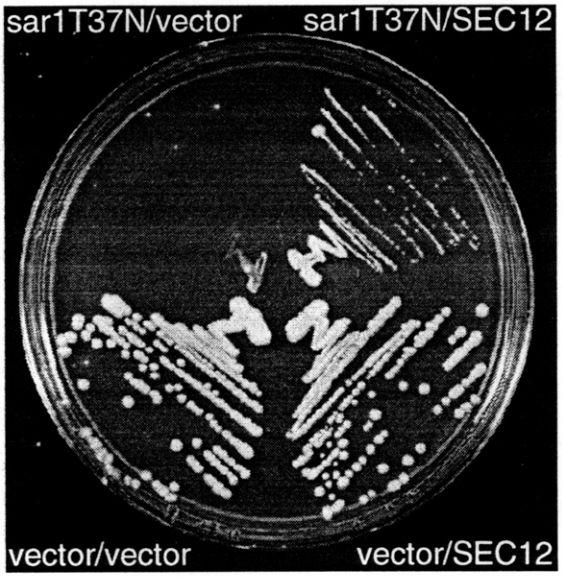

GST-Sec16 (1892-2194)
GST-Sec16 (1892-2095)
GST
B S B S B S
Sec23p
1 2 3 4 5 6

Figure 3. Overexpression of *SEC12* suppresses the toxicity of a Sar1p-GDP mutant, but not a Sar1p-GTP mutant. Yeast strains, derived from CKY473, overexpressing either (a) a GDP-restricted mutant of Sar1p (*sar1T37N*) or (b) a GTP-restricted mutant of Sar1p (*sar1H77G*), and *SEC12* from the *GAL* promoter were plated for single colonies on media containing 2% galactose. Control strains were transformed with the vectors pRS315 and pRS316. Cells were incubated at 30˚C for 4 days prior to photography.

a


sar1T37N/vector
sar1T37N/SEC12
vector/vector
vector/SEC12

b

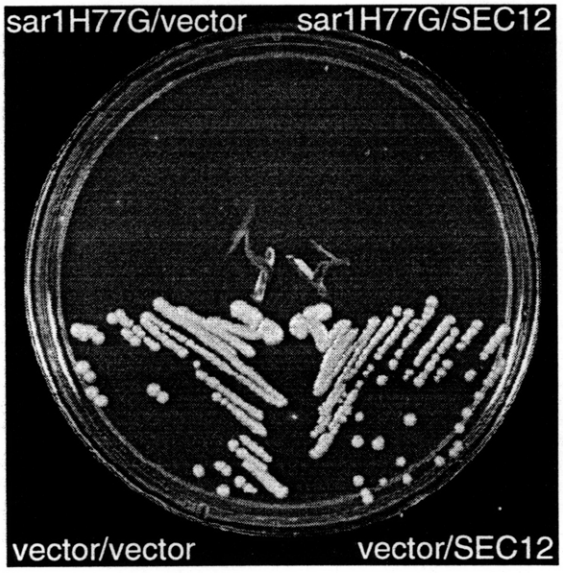
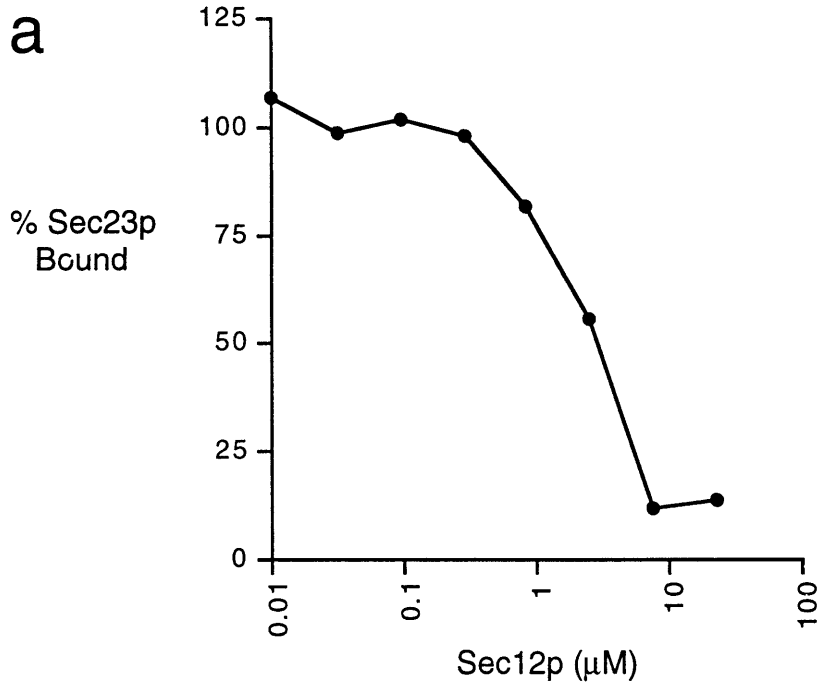

sar1H77G/vector
sar1H77G/SEC12
vector/vector
vector/SEC12

Figure 4. Sec12p binds directly to the COOH-terminus of Sec16p. Binding reactions contained one of the following GST fusion proteins immobilized on glutathione Sepharose beads: lanes 1 and 2, 60 pmol of yeast GST-Sec16p (aa 1892-2194); lanes 3 and 4, 15 pmol of bacterial GST-Sec16p (aa 1892-2095); lanes 5 and 6, 15 pmol of bacterial GST. To test binding, we incubated 1 pmol of recombinant Sec12p (aa 1-354) with the immobilized proteins for 1 hr at 22°C. One-tenth of the bound (B) and soluble (S) fractions were resolved by SDS-PAGE and Sec12p (aa 1-354) was detected by Western blotting with Sec12p antiserum.


GST-Sec16 (1892-2194)
GST-Sec16 (1892-2095)
GST
B S B S B S
Sec12p
1 2 3 4 5 6

Figure 5. Sec12p and Sec23p compete for binding to the COOH-terminus of Sec16p. All binding reactions contained 8 pmol of yeast GST-Sec16p (aa 1892-2194) immobilized on glutathione Sepharose beads. (a) Binding of Sec23p (0.2 μM, 1 pmol) to GST-Sec16p (aa 1892-2194) was assayed in the presence of increasing concentrations of Sec12p (aa 1-354). (b) Binding of Sec12p (aa 1-354) (0.2 μM, 1 pmol) to GST-Sec16p (aa 1892-2194) was assayed in the presence of increasing concentrations of Sec23p. The amount of Sec23p (a) or Sec12p (aa 1-354) (b) bound to the beads in the absence of competing protein was normalized to 100%. The fraction of Sec23p or Sec12p (aa 1-354) bound to GST-Sec16p (aa 1892-2194) in the absence of competing protein is shown in Figure 2 (lanes 1 and 2) and Figure 4 (lanes 1 and 2). Sec23p and Sec12p (aa 1-354) were resolved by SDS-PAGE and detected by Western blotting with Sec23p and Sec12p antisera.


a
125
100
75
50
25
0
% Sec23p Bound
0.01
0.1
1
10
100
Sec12p (μM)

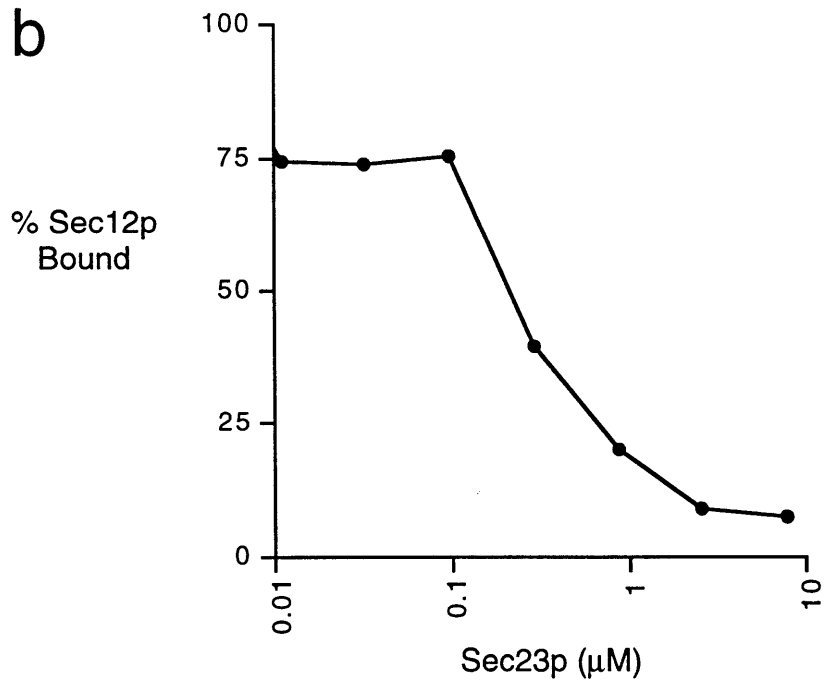

b
100
75
50
25
0
% Sec12p Bound
0.01
0.1
1
10
Sec23p (μM)

Figure 6. Model for the regulation of vesicle formation. Sec12p is bound to Sec16p on the ER membrane through an interaction with the COOH-terminus of Sec16p. *Step 1*, this complex recruits Sar1p and activates Sar1p by exchanging GDP for GTP. *Step 2*, activated Sar1p recruits Sec23p to bind to Sec16p, which displaces Sec12p. Assembly of the remaining COPII components facilitates vesicle formation. *Step 3*, Sec23p catalyzes hydrolysis of GTP by Sar1p. *Step 4*, formation of Sar1p-GDP weakens the interaction between Sec16p and Sec23p and leads to coat disassembly.

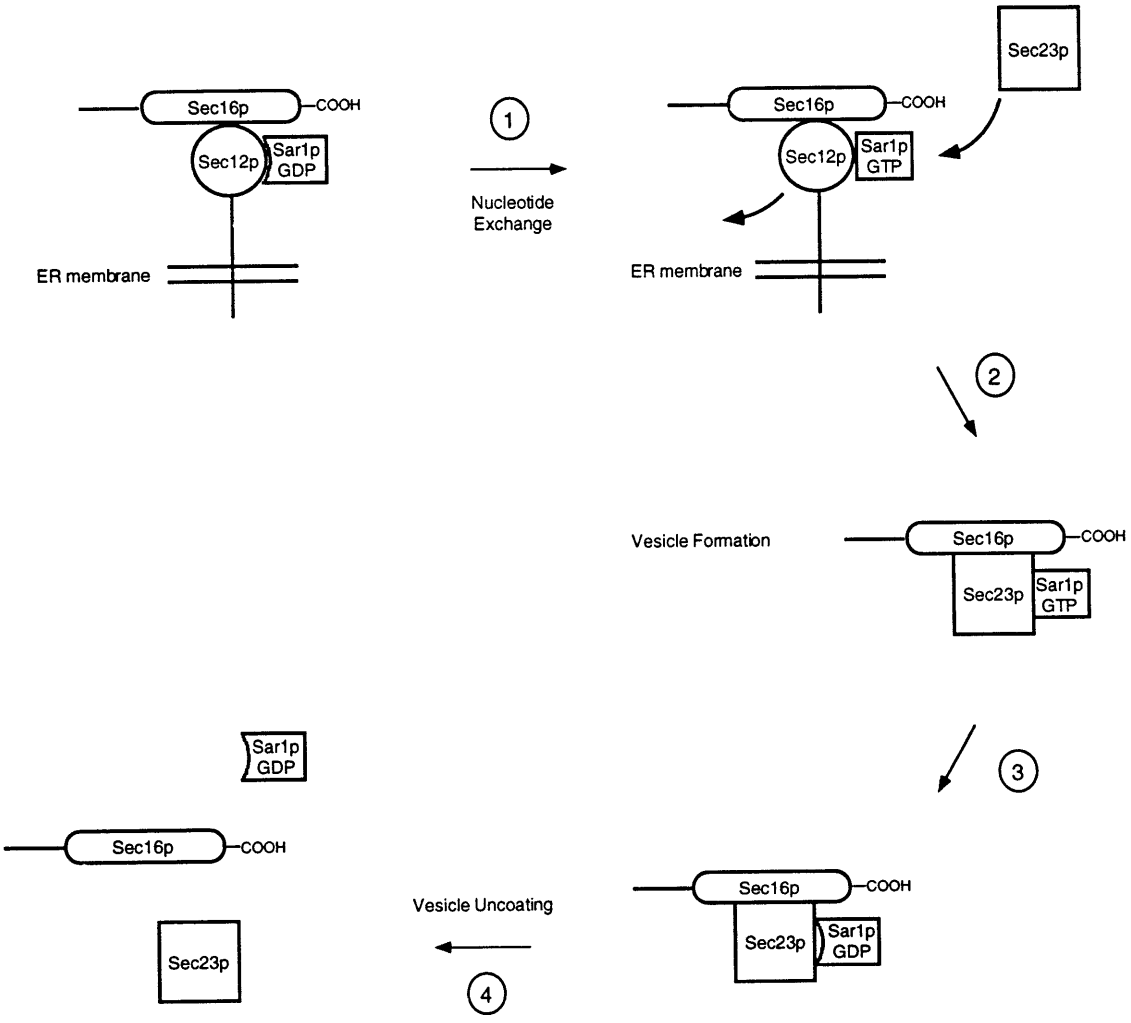

Sec16p
COOH
Sec12p
Sar1p
GDP
ER membrane
1
Nucleotide
Exchange
Sec23p
Sec16p
COOH
Sec12p
Sar1p
GTP
ER membrane
2
Vesicle Formation
Sec16p
COOH
Sec23p
Sar1p
GTP
3
Sar1p
GDP
Sec16p
COOH
Sec23p
Vesicle Uncoating
4
Sec16p
COOH
Sec23p
Sar1p
GDP

Table I. Plasmids

Plasmid	Description	Source
pRS315	centromere vector marked with *LEU2*	Sikorski and Hieter, 1989
pRS316	centromere vector marked with *URA3*	Sikorski and Hieter, 1989
pPE10	*GAL1* promoted *SEC16-HA* in pRS315	This study
pPE239	*GAL1* promoted *SAR1* in pRS315	This study
pPE275	*GAL1* promoted *sar1T37N* in pCD43	This study
pPE276	*GAL1* promoted *sar1H77G* in pCD43	This study
pPE262	*GAL10* promoted *SEC12* in pRS315	This study
pCD43	*GAL1/GAL10* promoter in pRS316	Shaywitz et al., 1995
pPE255	*SEC16 (aa 1892-2194)* in pRD56	This study
pRD56	*GAL1* promoted *GST* in pRS316	Espenshade et al., 1995
pPE223	*SEC12 (aa 1-354)* in pGEX-2T	This study
pPE271	*SEC16 (aa 1892-2095)* in pGEX-2T	This study
pPE124	*SEC23* in pGEX-2T	Gimeno et al., 1996
pPE229	*SEC23-6x His* in pGEX-2T	This study
pGEX-2T	bacterial GST expression vector	Smith and Johnson, 1988

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

Chapter V

Self-Association of Sec16p Molecules is Mediated by a Domain Containing the *sec16^{ts}* Mutations

Abstract

Prior studies have shown that Sec16p is an insoluble, 240 kD peripheral ER membrane protein that binds to the COPII proteins Sec23p, Sec24p, and Sec31p, and that Sec16p is a component of the COPII coat (Espenshade *et al.*, 1995; Gimeno *et al.*, 1996; Shaywitz *et al.*, 1997). In addition, genetic experiments have shown that *SEC16* is required for vesicle formation from the ER in vivo (Kaiser and Schekman, 1990; Espenshade *et al.*, 1995). These data have led to the model that Sec16p functions to organize the assembly of the soluble COPII proteins on the ER membrane during vesicle formation. In this report, we examined the ability of Sec16p to bind to itself. Sec16p was shown to self-associate in both copurification experiments using yeast cell extracts and the two-hybrid assay. A 460 amino acid domain located in the central region of the protein is sufficient to promote this interaction. Interestingly, each of the four *SEC16* temperature-sensitive mutations is located within this region. Two-hybrid studies using these temperature-sensitive alleles of *SEC16* showed that the mutant proteins are defective for self-association, suggesting that the ability of Sec16p to oligomerize may be important for proper vesicle formation in vivo.

Introduction

Transport of secretory proteins from the endoplasmic reticulum (ER) to the Golgi apparatus in the yeast *Saccharomyces cerevisiae* is mediated by vesicle intermediates that are formed by the assembly of cytosolic coat proteins onto the ER membrane (Schekman and Orci, 1996). Three cytoplasmic factors, collectively termed COPII (Sar1p, Sec23p/Sec24p, and Sec13p/Sec31p), can substitute for cytosol in a reaction that reconstitutes vesicle formation in vitro using urea-washed ER membranes (Barlowe *et al.*, 1994). These proteins form a coat around the vesicle, hence the name COPII vesicles. While the minimal, soluble components needed for this reaction have been identified, the necessary membrane associated factors are less well defined.

A growing body of evidence identifies Sec16p as a membrane component that is essential for vesicle formation. *SEC16* is required for transport vesicle formation in vivo and displays genetic interactions with each of the five COPII genes (Kaiser and Schekman, 1990). *SEC16* codes for a 240 kD insoluble, ER peripheral membrane protein that can be solubilized by high salt, but not extracted from membranes by urea (Espenshade *et al.*, 1995). Furthermore, Sec16p is a component of the COPII coat since membrane bound Sec16p is incorporated into vesicles formed in vitro (Espenshade *et al.*, 1995). ER membranes prepared from *sec16* mutant strains are defective for the release of ER-derived vesicle cargo proteins in vitro (Campbell and Schekman, 1997). Finally, in vitro binding studies have shown that Sec16p binds to the COPII proteins, Sec23p, Sec24p and Sec31p (Espenshade *et al.*, 1995; Gimeno *et al.*, 1996; Shaywitz *et al.*, 1997). Collectively, these data support a model in which Sec16p acts as a membrane bound receptor on the ER to organize the assembly of the soluble COPII proteins during vesicle formation.

Polymerization of COPII subunits is believed to cause deformation of the

ER membrane and subsequent vesicle formation (Schekman and Orci, 1996). In addition to the interactions between COPII subunits and Sec16p, Sec13p/Sec31p and Sec23p/Sec24p can bind to each other (Shaywitz *et al.*, 1997). Before the COPII subunits can bind to one another, these proteins must be localized to the same region of the ER membrane. One possible way to concentrate COPII subunits at the ER membrane and facilitate COPII subunit interactions would be to have Sec16p molecules pre-existing on the membrane in a lattice-like array. To investigate the potential for Sec16p to form a lattice-like structure, we examined the ability of Sec16p to self-associate. In this paper, we demonstrate that Sec16p can oligomerize and that this oligomerization is mediated by the central region of the protein that contains each of the four *SEC16* temperature-sensitive mutations.

Materials and Methods

General 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 stated, cultures were grown in synthetic complete medium with 2% glucose and with supplements appropriate for selection. DNA manipulations were performed using standard techniques (Sambrook *et al.*, 1989). PCR was performed using Vent polymerase according to the manufacturer's specifications (New England Biolabs, Beverly, MA). Western blotting was performed as described (Espenshade *et al.*, 1995) with the following antibodies: anti-HA 12CA5 monoclonal (1:1000, BAbCO, Richmond, CA) and anti-mouse IgG, peroxidase coupled whole antibody from sheep (1:10,000, Amersham Corp., Arlington Heights, IL); anti-invertase (1:1,000) and anti-GST (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-rabbit IgG, peroxidase coupled whole antibody from donkey (1:10,000, Amersham Corp., Arlington Heights, IL). Western blots were developed using chemiluminescence (Pierce, Rockford, IL).

Construction of Chromosomal *GSTSEC16*

To analyze the association of proteins with Sec16p, a strain was constructed that contained a fusion of *SEC16* to the COOH-terminus of glutathione-*S*-transferase (GST) as its only allele of *SEC16* (Smith and Johnson, 1988). This gene fusion was targeted to the *SEC16* chromosomal locus and was expressed under the control of the *SEC16* promoter. This strain was constructed in a multi-step procedure. First, a gene fusion was made that contained 1 kb of *SEC16* 5' untranslated sequence fused to the initiator ATG of GST by means of splice overlap extension PCR (Taylor, 1991). The splice

overlap extension PCR used pPE5 as a template for primers 1 and 2 (Primer 1: 5'-GCATCTCGAGGAGCCCTACTTTCC - 3', primer 2: 5'-TATAGGGGACATTC-TTAACACGTTATTAAATGA-3') and pPE127 as a template for primers 3 and 4 (Primer 3: 5'-AACGTGTTAAGAATGTCCCCTATACTAGGT-3', Primer 4: 5'-TCT-GGATCCGATCCACGCGGAACCAG-3'). The product of the splice overlap extension PCR was digested with BamHI/XhoI (underlined) and ligated to a 6.6 kb BamHI fragment from pPE4 and to pRS306 digested with BamHI/XhoI to create pPE144. A *sec16-2* strain, CKY200, was transformed with pPE144 linearized in the 5' untranslated region of *SEC16* by digestion with ClaI to create a strain with the *GSTSEC16* allele integrated upstream of the *sec16-2* allele. Cells that had lost the *sec16-2* allele along with the integrating plasmid by recombination were selected by growth on 5-fluoro-orotic acid (Rothstein, 1991). A strain, PEY264, that had successfully lost the integrating plasmid and retained the *GSTSEC16* allele was identified by PCR and confirmed by DNA sequencing of PCR products. PEY270, a protease deficient derivative of PEY264, was made by disruption of the *PEP4* gene using the plasmid pTS15 as described (Ammerer *et al.*, 1986).

Construction of GST Fusions

A series of deletions of *SEC16* fused to GST under the control of the *GAL1* promoter were constructed. Plasmids pPE122, pRH323, pE127, and pRD56 have been described previously (see Table II). A fusion of *SEC16* to GST, pPE135, was made by inserting a 6.6 kb BamHI fragment from pPE4 into pPE127. A fusion of *SEC16* (aa 1017-2194) to GST, pPE149, was generated by inserting a 4.5 kb BamHI fragment from pPE46 into pRD56. A PCR fragment generated using pPE4 as a template and oligonucleotide primers (5'-TTGGAT-CCATGATTTCATCAAGCATTGTAC-3'; 5'-GAATTCCTAATTATGCAAAAGA-

GCTG-3') was digested with EcoRI/BamHI (underlined) and inserted into pRD56 to yield pPE171, a fusion of GST to *SEC16* (aa 1017-1474). Plasmid pPE51 used in the Sec16p binding experiments was a derivative of pPE46 that contained three tandem copies of the influenza HA epitope inserted at amino acid 1892 of Sec16p.

Construction of *SEC16* Two-Hybrid Plasmids

Fusions of Sec16p (aa 1017-1474) or Sec16p (aa 1017-1235) to either the *lexA* DNA binding domain in pEG202 or an acidic activation domain in pJG4-5 were made by inserting an EcoRI/XhoI (underlined) digested PCR fragment into the appropriate plasmid. PCR products were generated using pPE4 as a template and the following oligonucleotide primers: 5'-TTGAATTCA-TGATTTCATCAAGCATTGTAC-3' and 5'-GCCGCTCGAGCTAATTATGCAAAA-GAGCTG-3'(aa 1017-1474) or 5'-GCCGCTCGAGCTAAGCTGCAACGATACTC-TT-3'(aa 1017-1235). Plasmids encoding Sec16p (aa 878-1474) in pEG202 and pJG4-5 were created by inserting an EcoRI/XhoI (underlined) digested PCR product generated using template DNA containing the appropriate genomic *SEC16* allele and oligonucleotide primers (5'-CGGAATTCAATTCAAGCTATCA-AAGC-3', 5'-GCCGCTCGAGCTAATTATGCAAAAGAGCTG-3').

Affinity Isolation of GST-Sec16p

Cultures of the strains PEY271 and PEY205 were grown at 30°C to exponential phase in synthetic medium containing 2% glucose. 2 x 10^8 cells were spheroplasted and then gently lysed with glass beads in extraction buffer (20 mM Hepes pH 6.8, 80 mM KOAc, 5 mM MgOAc, 0.02% Triton X-100) containing the protease inhibitors leupeptin (0.5 μg/ml), aprotinin (2 μg/ml), pepstatin (0.7 μg/ml) and PMSF (1 mM). The lysate was diluted to 1 ml with

extraction buffer and membranes were collected by centrifugation at 2,000 *g* for 10 min at 4˚C. Membranes were resuspended in 333 µl of extraction buffer containing 0.6 M NaCl to solubilize Sec16p and the sample was incubated for 30 min on ice. Membranes were removed from the lysate by centrifugation at 16,000 *g* for 10 min at 4˚C. The resulting supernatant was diluted to 1 ml with extraction buffer containing 0.6 M NaCl and incubated with 15 µl of packed Glutathione Sepharose 4B beads (Pharmacia Biotech, Piscataway, NJ) for 1 hr at 4˚C. Beads were washed three times with 1 ml of extraction buffer containing 0.6 M NaCl. Proteins were solubilized in sample buffer and resolved by SDS-PAGE. Proteins bound to the GST-Sec16p fusion proteins were detected by Western blotting using either the 12CA5 mAb.

Self-association of Sec16p fragments was tested in extracts from PEY207 transformed with a *GSTSEC16* expression plasmid and another plasmid expressing a fragment of Sec16p containing an epitope tag. Expression of both proteins was controlled by the *GAL1* promoter. Cells were grown at 30˚C to exponential phase in synthetic medium containing 2% raffinose, and expression of the Sec16p proteins was induced for 2 hours by the addition of 2% galactose. Cell lysates were processed as described above with the following exceptions. Extracts were prepared from 10^8 cells. Following the removal of membranes at 16,000 *g*, the lysate was diluted using extraction buffer. Subsequent washes of the beads were performed using extraction buffer containing 0.2 M NaCl. Proteins were detected by Western blotting using either the 12CA5 mAb or antibodies to invertase.

Two-Hybrid Protein-Protein Interaction Assay

Potential interactions between Sec16p fragments were investigated using the two-hybrid protein interaction assay and the plasmids pEG202, pJG4-

5, and pSH18-34 (Gyuris *et al.*, 1993). Fragments of Sec16p were expressed either as fusions to the *lexA* DNA binding domain in pEG202 or as fusions to an acidic transcriptional activator in pJG4-5. Combinations of control or *SEC16* fusion plasmids were transformed into the strain EGY40 (Golemis and Brent, 1992). Positive interactions were scored visually by colony assay or quantitatively by β-galactosidase assay after growth at 30˚C as described (Espenshade *et al.*, 1995).

Results

GST-Sec16p binds to Sec16p-HA

To test for the ability of Sec16p to self-associate, a yeast strain was constructed in which the chromosomal *SEC16* allele was replaced by *GSTSEC16*, which codes for a fusion of glutathione S-transferase and Sec16p under the control of the *SEC16* promoter. In yeast cell lysates, Sec16p is insoluble in buffer containing 0.1 M NaCl, but Sec16p can be solubilized by treatment of membranes with high salt (Espenshade *et al.*, 1995). Membranes isolated from strains expressing either *GSTSEC16* or *SEC16* and epitope-tagged *SEC16-HA* from a plasmid were treated with 0.6 M NaCl to solubilize the Sec16p molecules. Proteins bound to GST-Sec16p were recovered on glutathione Sepharose beads following washes with buffer containing 0.6 M NaCl, and the presence of Sec16p-HA was examined by Western blotting with the 12CA5 mAb. Sec16p-HA was recovered from a strain expressing *GSTSEC16*, but not *SEC16* (Fig. 1), demonstrating that GST-Sec16p forms a complex with Sec16p-HA in yeast cell extracts. Furthermore, the binding between GST-Sec16p and Sec16p-HA was stable in 0.6 M NaCl buffer, suggesting that Sec16p molecules are tightly associated on the membrane.

Mapping of the Sec16p Self-Association Domain

To identify the region(s) of Sec16p responsible for self-binding, deletions of *SEC16,* fused to GST and expressed from the *GAL1* promoter, were tested for binding to epitope tagged fragments of Sec16p, also expressed from the *GAL1* promoter. Sec16p proteins bound to membranes from cell lysates were solubilized with 0.6 M NaCl and the salt concentration was reduced to 0.2 M NaCl. GST-Sec16p proteins were isolated using glutathione Sepharose and binding of the epitope-tagged Sec16p protein was assayed by Western blotting

with the appropriate antibody. Sec16p-HA (aa 1017-2194) bound to both full-length GST-Sec16p and to GST-Sec16p (aa 1017-2194), but not to GST-Sec16p (aa 1638-2194) or to GST alone (Fig. 2a). These results suggested that the NH_2-terminus was not required for binding and established an NH_2-terminal boundary for the oligomerization domain at amino acid 1017.

SEC16 (aa 565-1235) fused to *SUC2*, encoding cytoplasmic invertase, bound to GST-Sec16p (aa 565-1235), but not to GST-Sec16p (aa 1638-2194) (Fig. 2b) These data suggested that the COOH-terminus of Sec16p was not required for binding and placed the COOH-terminal boundary of the binding domain at amino acid 1235. In the final set of binding tests, GST-Sec16p (aa 1017-1474) bound to both Sec16p-HA (aa 1017-2194) and to Sec16p (aa 565-1235)-SUC2, demonstrating that amino acids 1017-1474 were sufficient for binding (Fig. 2c). Collectively, these results mapped the oligomerization domain to the central region of Sec16p and suggested that amino acids 1017-1235 of Sec16p may be sufficient for self-association.

To confirm the binding interactions observed in yeast extracts, we examined the self-association of Sec16p using the two-hybrid assay (Gyuris *et al.*, 1993). Three deletions of *SEC16* (aa 1017-1474; aa 1017-1235; aa 447-1737) were tested independently by fusion to the *lexA* DNA binding domain. *SEC16* (aa 1017 -1474) and *SEC16* (aa 1017-1235) were fused to an acidic activation domain and binding to each of the three *SEC16* deletions was tested by assaying activation of transcription of a *lacZ* reporter gene. A strong interaction was observed between the two *SEC16* (aa 1017-1474) fusions, but no interaction was seen between the *SEC16* (aa 1017-1474) and *SEC16* (aa 1017-1235) fusions (Fig. 3a). Consistent with this observation, no binding was detected between the two *SEC16* (aa 1017-1235) fusions. Interestingly, both *SEC16* (aa 1017-1474) and *SEC16* (aa 1017-1235) showed a strong

interaction with a larger fusion, *SEC16* (aa 447-1737) (Fig. 3a). These data were entirely consistent with those obtained from the experiments using cell extracts, and demonstrated that amino acids 1017-1474 can function as a minimal oligomerization domain. Furthermore, amino acids 1017-1235 can function as a binding site when tested against a larger fragment of Sec16p (aa 447-1737).

sec16^{ts} Mutations Affect Oligomerization of Sec16p

Deletion mapping studies identified amino acids 1017-1474 as sufficient to mediate oligomerization of Sec16p molecules. Four temperature-sensitive mutations have been isolated in *SEC16* that block ER to Golgi transport in vivo at the non-permissive temperature (Espenshade *et al.*, 1995). These mutations map to the region of Sec16p that is sufficient for oligomerization; *sec16-1* (aa 1230), *sec16-2* (aa 1088), *sec16-3* (aa 1083), and *sec16-4* (aa .058). To investigate whether the *sec16^{ts}* mutations affect Sec16p oligomerization, three alleles (*SEC16*, *sec16-1*, and *sec16-2)* were assayed in pairwise tests for self-association of Sec16p (aa 878-1474) using the two-hybrid assay. Each of the two-hybrid fusion proteins tested differed only by a single amino acid.

A strong interaction was detected when both wild-type alleles of *SEC16 (aa 878-1474)* were tested (Fig. 3b). The strength of the interaction decreased if only one of the tested alleles was wild-type and the other was either *sec16-1* or *sec16-2*. A further decrease in the measured β-galactosidase activity was seen if both alleles tested were mutant. The weakest interaction recorded was between *sec16-1 (878-1474)* and *sec16-1 (878-1474)* which showed greater than a 100x lower level of β-galactosidase activity when compared to the *SEC16/SEC16* interaction. These data demonstrate that fragments of Sec16p containing the *sec16-1* and *sec16-2* mutations are defective for self-association

in the two-hybrid assay and suggest that oligomerization of Sec16p may be required for proper ER to Golgi transport in vivo. However, it is also possible that the *sec16*^{ts} mutations lead to reduced levels of the two-hybrid fusion proteins, resulting in less transcriptional activation of the *lacZ* reporter gene.

Discussion

Previous biochemical and genetic studies have shown that Sec16p is a 240 kD, peripheral ER membrane protein that binds to the COPII proteins Sec23p, Sec24p, and Sec31p, and is required for vesicle formation in vivo (Espenshade *et al.*, 1995; Gimeno *et al.*, 1996; Shaywitz *et al.*, 1997). Formation of the vesicle coat is thought to require the polymerization of COPII subunits in a localized area of the ER membrane (Schekman and Orci, 1996). These data have led to the model that Sec16p organizes the assembly of the soluble COPII proteins on the ER membrane during vesicle formation. Sec16p could function as an organizer by simply providing a lattice of COPII binding sites on the ER. In this report, we investigated this hypothesis by testing whether Sec16p can bind to itself.

In yeast cell extracts, Sec16p molecules are insoluble in buffer containing 0.1 M NaCl, but can be solubilized by the addition of 0.5 M NaCl (Espenshade *et al.*, 1995). In this study, we demonstrate that Sec16p molecules, solubilized from membranes, form a complex even in the presence of 0.6 M NaCl, indicating that the Sec16p can bind to itself and that this complex is stable in high salt. The number of Sec16p molecules in this complex is not known. However, an attempt was made to estimate the size of this salt extracted complex using gel filtration chromatography. A complex containing Sec16p-HA, extracted from membranes in 0.6 M NaCl, fractionated in the void volume of an Sephacryl S-300 column (exclusion limit of 1.5×10^6 Da for globular proteins) and in the included volume of an Sephacryl S-1000 column (exclusion limit of 8×10^6 Da for globular proteins) (Espenshade and Kaiser, unpublished observations). Together, these data suggest that salt-solubilized Sec16p exists in a stable high molecular weight complex of approximately 1.5 - 8×10^6 Da.

Deletion studies mapped the domain sufficient for oligomerization to amino acids 1017-1474 of Sec16p. A priori, the binding sites did not have to be in the same region of the protein. For example, binding of two Sec16p molecules could occur through the binding of the NH_2-terminus of one molecule to the COOH-terminus of another molecule. However, the data indicate that a single domain of Sec16p can function as the binding site in each Sec16p molecule. A smaller oligomerization domain of Sec16p (aa 1017-1235) was defined by experiments using cell extracts, but this fragment was unable to bind to itself in the two-hybrid assay. In contrast, this fragment could bind to a larger fusion protein containing aa 447-1737 of Sec16p, suggesting that the binding properties of amino acids 1017-1235 may be sensitive to the protein environment. For instance, amino acids 1017-1235 might represent the true minimal oligomerization domain, but flanking sequences may be required for the proper folding of this domain.

Each of the four temperature-sensitive mutations in *SEC16* is located between amino acids 1017-1235. The effect of these mutations on the self-association of Sec16p was tested using the two-hybrid assay. Tests between the mutant fusion proteins gave lowered levels of β-galactosidase activity when compared to the wild-type fusion proteins, indicating a defect in Sec16p binding. Thus, it is possible that the temperature-sensitive defect in Sec16p is due to the inability of Sec16p to oligomerize, and that oligomerization of Sec16p may be critical for the formation of ER to Golgi transport vesicles. However, no defect in the binding of full-length mutant GST-Sec16p molecules to full-length mutant Sec16p-HA molecules was observed in yeast cell extracts, even under conditions of 1 M NaCl (Espenshade and Kaiser, unpublished observation). It is possible that other oligomerization domains may exist in Sec16p since the deletion analysis only identified a minimal region that was

sufficient for self-association. If this is the case, the *sec16* point mutations may result in a localized disruption in the central domain that inhibits oligomerization. Alternatively, the mutations may destabilize the fusion proteins and lead to lowered protein levels which would also result in reduced β-galactosidase activity.

Three proteins are now known to bind to amino acids 447-1235 of Sec16p: Sec16p, Sec24p, and Sec31p (Gimeno *et al.*, 1996; Shaywitz *et al.*, 1997). Together with the clustered nature of the temperature-sensitive mutations, these binding properties point to a role for the central region of Sec16p in the regulation of the assembly of COPII proteins. Experiments described in this paper suggest that the oligomerization of Sec16p may be an important step in the regulation of formation of COPII vesicles. Further experiments using mutant Sec16p proteins and the COPII proteins may lead to a better understanding of this regulation and uncover nature of the defect in *SEC16* temperature-sensitive mutants.

The new evidence that Sec16p can oligomerize into a high molecular mass structure on the ER membrane provides an explanation for how the stepwise assembly of soluble COPII proteins onto Sec16p may be linked to the mechanism of vesicle budding. In this model, Sec23p is recruited to the ER membrane by Sar1p-GTP and binds to the COOH-terminus of Sec16p. Next, Sec24p, complexed to Sec23p, binds to the central domain of Sec16p. Sec13p/Sec31p complex would then be recruited to the membrane by virtue of the affinity of Sec31p for binding to Sec16p, Sec23p, and Sec24p (Shaywitz *et al.*, 1997). The presence of oligomerized Sec16p on the membrane allows multiple COPII subunits to bind in a localized region. Consequently, interactions between COPII subunits bound to different Sec16p molecules could initiate the polymerization of the coat that results in COPII vesicle

budding. In this model, Sec16p serves as the foundation for the construction of the vesicle coat. Efforts to isolate such a "pre-budding" protein complex have been difficult due to the insolubility of Sec16p and the sensitivity of the COPII binding interactions to high concentrations of salt. However, the availability of purified COPII proteins and soluble fragments of Sec16p may soon permit the reconstitution of this complex in vitro.

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Figure 1. GST-Sec16p binds to Sec16p-HA. Lanes 1 and 2: PEY271, with both *GSTSEC16* and *SEC16-HA* (pPE26). Lanes 3 and 4: PEY205, with *SEC16* and *SEC16-HA* (pPE26). Expression of proteins was controlled by the *SEC16* promoter. Membranes from 2 x 10^8 cells were collected at 2,000 *g* and treated with buffer containing 0.6 M NaCl to solubilize Sec16p. The cleared extract was incubated with glutathione Sepharose beads which were washed with buffer containing 0.6 M NaCl. Proteins bound to glutathione beads are in lanes 2 and 4. Five percent of the total lysate was loaded in the extract lanes 1 and 3. Sec16p-HA and GST-Sec16p were detected by SDS-PAGE and Western blotting using the 12CA5 mAb and antibodies to GST, respectively.

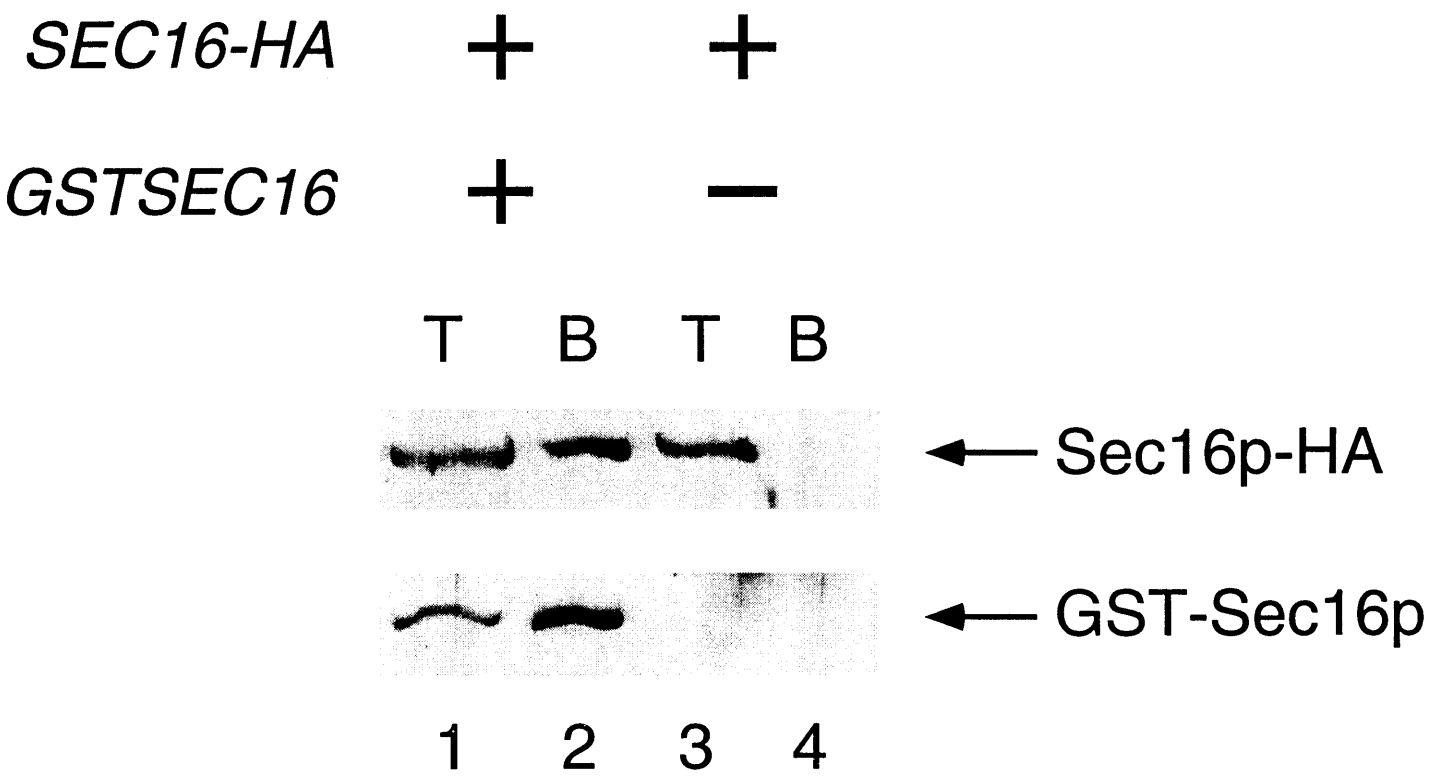
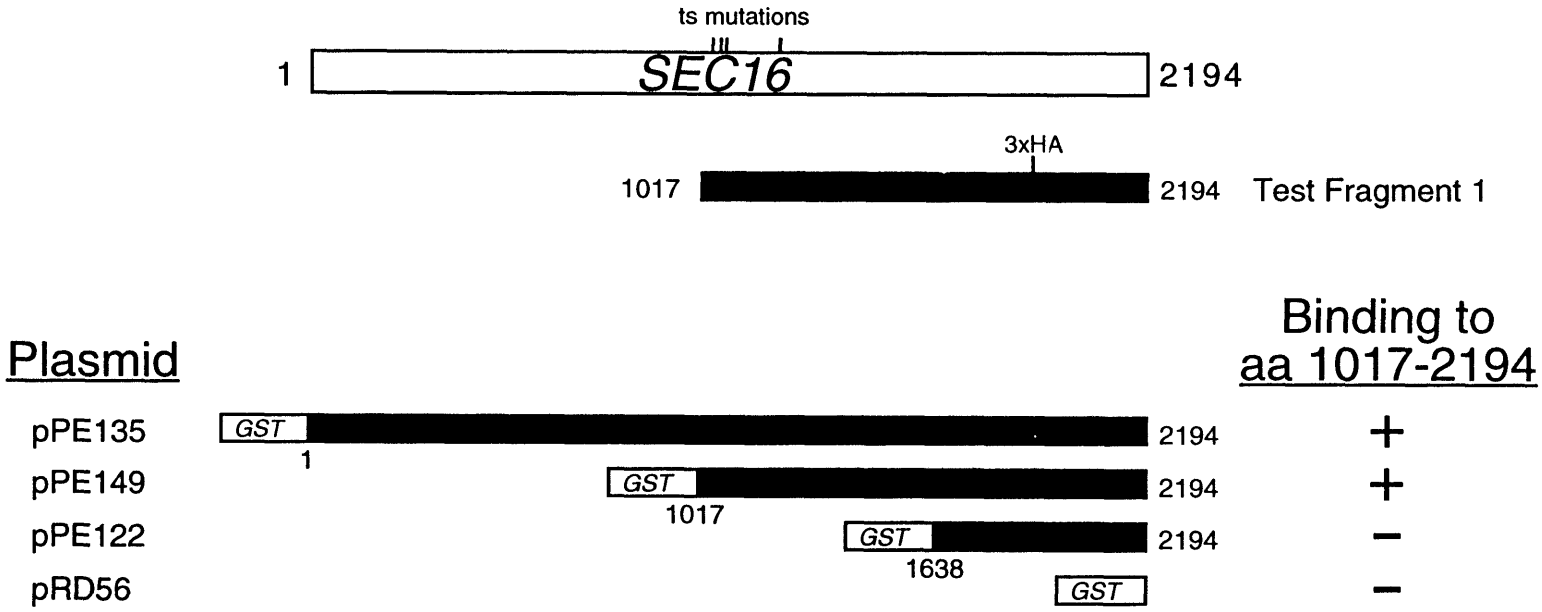
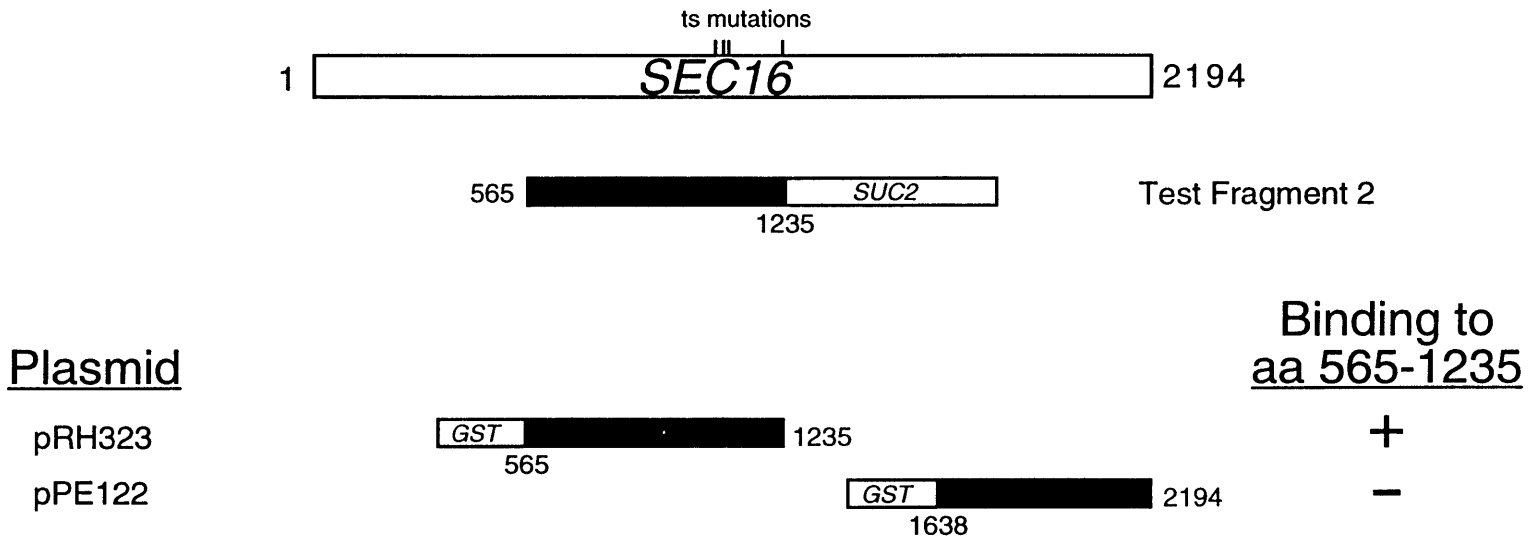

SEC16-HA + +
GSTSEC16 + −
T B T B
Sec16p-HA
GST-Sec16p
1 2 3 4

Figure 2. Mapping of the Sec16p self-association domain. (a) Binding of GST-Sec16p, GST-Sec16p (aa 1017-2194), GST-Sec16p (aa 1638-2194), and GST to Sec16p-HA (aa 1017-2194). (b) Binding of GST-Sec16p (aa 565-1235) and GST-Sec16p (aa 1638-2194) to Sec16p (aa 565-1235) fused to invertase. (c) Binding of GST-Sec16p (aa 1017-1474) to Sec16p-HA (aa 1017-2194) and Sec16p (aa 565-1235) fused to invertase. Binding of GST-Sec16p fusion proteins to fragments of Sec16p was assayed in cell extracts as described in Materials and Methods. A positive interaction (+) indicates significant levels of binding (1%-5% of total) above the background with GST alone. Dark bars represent *SEC16* sequences and the amino acid endpoints are given.

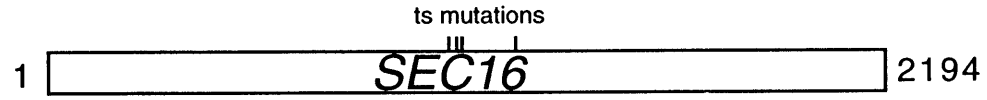
a


ts mutations
1
SEC16
2194
3xHA
1017
2194
Test Fragment 1
Plasmid
Binding to aa 1017-2194
pPE135
GST
1
2194
+
pPE149
GST
1017
2194
+
pPE122
GST
1638
2194
−
pRD56
GST
−

b


ts mutations
1
SEC16
2194
565
SUC2
1235
Test Fragment 2
Plasmid
Binding to
aa 565-1235
pRH323
GST
1235
565
+
pPE122
GST
2194
1638
−

C



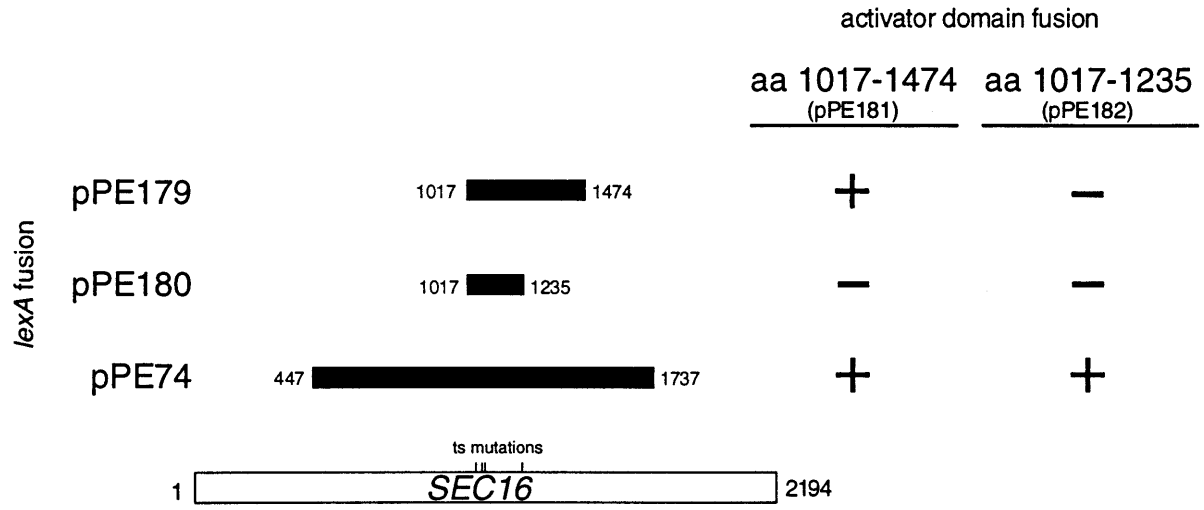
Test Fragment 3

Plasmid		Binding to aa 1017-1474
pPE51	1017 — 3xHA — 2194	+
pRH317	565 — SUC2 — 1235	+

Sec16p Oligomerization Domain ?

Figure 3. Identification of the Sec16p self-association domain using two-hybrid analysis. (a) A series of Sec16p deletions fused to the *lexA* DNA binding domain [Sec16p (aa 1017-1474); Sec16p (aa 1017-1235); Sec16p (aa 447-1737)] were assayed against fragments of Sec16p fused to an acidic activation domain [Sec16p (aa 1017-1474); Sec16p (aa 1017-1235)] for activation of expression of a *lacZ* reporter gene. Strains containing a combination of control or Sec16p plasmids were plated on indicator media at 30°C. A positive interaction (+) was scored as the appearance of a strong, blue color in the colony relative to control strains. Amino acid numbers are given for the *SEC16* sequence. (b) The effect of *sec16* temperature sensitive mutations on the self-interaction of Sec16p (aa 878-1474) fused to both a *lexA* DNA binding domain and an acidic activation domain was assayed at 30°C. Units of β-galactosidase activity (nmol/mg x min) were calculated as (optical density at 420 nm x vol. of assay)/0.0045 x concentration of protein in extract assayed x time). Activities shown were the mean ± SD for three independent transformants.

a



b

activator domain fusion

lexA fusion	*SEC16* (pPE293)	*sec16-1* (pPE294)	*sec16-2* (pPE295)
SEC16 (pPE290)	883 ± 62	80 ± 6	59 ± 16
sec16-1 (pPE291)	66 ± 9	8 ± 0	15 ± 11
sec16-2 (pPE292)	22 ± 4	9 ± 2	10 ± 2

β-galactosidase activity

Table I. *S. cerevisiae* strains

Strain	Genotype	Source
CKY93	*MATα ura3-52 leu2-3,112 pep4::URA3*	Kaiser Lab Collection
CKY200	*MATa sec16-2 ura3-52 leu2-3,112 his4-619* Gal$^+$	Kaiser Lab Collection
CKY473	*MATα ura3-52 leu2-3,112* Gal$^+$	Kaiser Lab Collection
PEY205	CKY93 (pPE26)	This study
PEY264	*MATa ura3-52 leu2-3,112 his4-619 sec16::GSTSEC16* Gal$^+$	This study
PEY270	*MATa ura3-52 leu2-3,112 his4-619 sec16::GSTSEC16 pep4::URA3* Gal$^+$	This study
PEY271	PEY270 (pPE26)	This study
EGY40	*MATα ura3-52 leu2 his3 trp1*	Golemis and Brent, 1992

Table II. Plasmids

Plasmid	Description	Source
pRS306	integrating vector marked with *URA3*	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
pPE4	*GAL1* promoted *SEC16* in pRS315	Espenshade *et al.*, 1995
pPE5	*SEC16* in YCp50	Gimeno *et al.*, 1995
pPE8	*SEC16* in pRS315	Espenshade *et al.*, 1995
pPE26	*SEC16-HA* in pRS315	Espenshade *et al.*, 1995
pPE46	*GAL1* promoted *SEC16(1017-2194)* in pRS315	Espenshade *et al.*, 1995
pPE51	*GAL1* promoted *SEC16-HA(1017-2194)* in pRS315	This study
pPE144	*SEC16GSTSEC16* in pRS306	This study
pRH317	*GAL1* promoted *SEC16(565-1235)-SUC2* in pRS315	Gimeno *et al.*, 1996
pRD56	*GAL1* promoted *GST* in pRS316	Espenshade *et al.*, 1995
pPE122	*GAL1* promoted *GSTSEC16(1638-2194)* in pRS316	Gimeno *et al.*, 1995
pPE127	*GAL1* promoted *GST* in pRS316	Gimeno *et al.*, 1996
pPE135	*GAL1* promoted *GSTSEC16* in pRS316	This study
pPE149	*GAL1* promoted *GSTSEC16(1017-2194)* in pRS316	This study
pPE171	*GAL1* promoted *GSTSEC16(1017-1474)* in pRS316	This study
pRH323	*GAL1* promoted *GSTSEC16(565-1235)* in pRS316	Gimeno *et al.*, 1996
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
pPE74	*SEC16(447-1737)* in pEG202	Espenshade *et al.*, 1995
pPE179	*SEC16(1017-1474)* in pEG202	This study
pPE180	*SEC16(1017-1235)* in pEG202	This study
pPE181	*SEC16(1017-1474)* in pJG4-5	This study
pPE182	*SEC16(1017-1235)* in pJG4-5	This study
pPE290	*SEC16(878-1474)* in pEG202	This study
pPE291	*sec16-1(878-1474)* in pEG202	This study
pPE292	*sec16-2(878-1474)* in pEG202	This study
pPE293	*SEC16(878-1474)* in pJG4-5	This study
pPE294	*sec16-1(878-1474)* in pJG4-5	This study
pPE295	*sec16-2(878-1474)* in pJG4-5	This study

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

Chapter VI

Prospectus

The focus of this dissertation has been the investigation of the role of *SEC16* in the formation of ER to Golgi transport vesicles. Genetic studies establish that *SEC16* is required for the formation of vesicles in vivo and that the stoichiometry of Sec16p is important for this function since overexpression of the protein blocks transport of proteins to the Golgi. Biochemical characterization of Sec16p reveals that the protein is a 240 kD insoluble, peripheral ER membrane protein that can be stripped from the membrane by high salt, but not by urea or non-ionic detergents. Analysis of purified transport vesicles synthesized in an in vitro budding assay indicates that Sec16p on the ER membrane is incorporated into transport vesicles as a component of the COPII vesicle coat.

A collective effort to examine the interactions between the soluble COPII proteins and membrane associated Sec16p demonstrates that Sec16p binds to the COPII coat proteins: Sec23p, Sec24p, and Sec31p (Gimeno *et al.*, 1996; Shaywitz *et al.*, 1997). The significance of these contacts in vivo is highlighted by genetic interactions between *SEC16* and genes encoding the COPII subunits (Kaiser and Schekman, 1990), and by the fact that a truncation mutant of Sec31p that is specifically deficient for binding to Sec16p is unable to support vesicle transport in vivo (Shaywitz et al, 1997). Together, this evidence indicates that Sec16p likely functions to organize the assembly of the soluble COPII coat proteins on the ER membrane during vesicle formation.

Recent data suggest that Sec16p may play an additional role in the regulation of the initiation of vesicle formation. The COOH-terminus of Sec16p binds directly to two proteins that control the GTPase cycle of Sar1p: Sec12p, an exchange factor, and Sec23p, a GTPase activating protein. Binding of Sec12p to Sec16p may create a high affinity binding site for Sar1p or possibly affect the rate of exchange of GTP for GDP on Sar1p. Either of these proposed

activities would result in the localized activation of Sar1p at the COOH-terminus of Sec16p. The recruitment of Sec23p to Sec16p by Sar1p would then initiate coat assembly (Fig. 7, Chapter 4). Consistent with this model, preliminary data indicate that Sar1p-GMP-PNP may stimulate the binding of Sec23p to Sec16p by altering the affinity of Sec23p for Sec16p. Collectively, these data suggest that binding of Sec16p to Sec12p might couple the activation of Sar1p to the binding of the COPII subunits to Sec16p on the ER membrane.

Sec16p oligomerizes on the ER membrane into a high molecular weight complex that can be removed by treatment with high salt. Indirect immunofluorescence experiments show that Sec16p localizes to the ER in a non-uniform, punctate pattern that may correspond to collections of these complexes. These patches of Sec16p on the membrane could potentially serve as the foundation for the formation of COPII coated vesicles. In this model, newly synthesized Sec16p assembles on the ER in a lattice-like complex that resembles a clathrin-coated pit on the plasma membrane (Fig. 1). In response to the activation of Sar1p, Sec23p/Sec24p is recruited to the ER membrane and binds to Sec16p followed by the binding of Sec13p/Sec31p to Sec16p. The oligomeric nature of Sec16p allows multiple COPII subunit complexes to bind to the same Sec16p lattice possibly through cooperative interactions with previously bound subunits. Polymerization of the coat leads to the formation of a vesicle containing a coat of Sec16p, Sec23p/Sec24p, Sec13p/Sec31p, and Sar1p. Identification of interactions between the Sec23p/Sec24p and Sec13p/Sec31p COPII subunits supports the idea that polymerization of COPII subunits leads to vesicle budding (Shaywitz *et al.* 1997). The isolation of purified Sec16p might allow the reconstitution of these assembly steps in vitro using purified COPII subunits.

The focus of this discussion has been on the mechanism by which the

COPII coat components are recruited to and bind to the ER membrane. Sec16p has been proposed to serve as a membrane receptor and possibly a regulator of this process. But how is Sec16p attached to the ER membrane? Chemical treatment experiments indicate that Sec16p is not an integral membrane protein, but that it is tightly bound to the membrane since it is released by buffers with high pH, but not by 2.5 M urea or Triton X-100. The ability of 0.5 M NaCl to extract Sec16p suggests that Sec16p may be bound to the membrane by ionic interactions, possibly with other ER resident proteins. Candidates for such proteins have come from a series of two-hybrid screens designed to identify proteins that bind to the COOH-terminus of Sec16p.

Screens looking for yeast proteins that bind to Sec16p (aa 1645-2194) identified eight proteins with at least one putative transmembrane domain (Gimeno, 1996; Shaywitz, 1997). Three of these proteins had been previously characterized, and are associated with the organelles of the secretory pathway: Chs1p, Hkr1p, and Ole1p. *CHS1* encodes a chitin synthase which contains three transmembrane domains and localizes to the plasma membrane (Leal-Morales *et al.*, 1994). *HKR1* encodes a type I transmembrane protein that also localizes to the plasma membrane and is required for proper cell wall function (Yabe *et al.*, 1996). Ole1p is an ER resident, fatty acid desaturase with seven potential transmembrane domains (Stukey *et al.*, 1990). Sec16p may bind to these proteins on the ER membrane and possibly assist in the transport of Chs1p and Hkr1p to the Golgi apparatus. Since Sec16p forms a homo-oligomeric complex on the ER membrane, multiple contacts between Sec16p molecules and resident ER proteins such as Ole1p or cargo proteins, such as Chs1p and Hkr1p, may result in the observed chemical properties of Sec16p. Further interaction studies will be required to determine if these proteins represent true binding partners for Sec16p on the ER membrane.

The mechanism of cargo selection in COPII vesicles is unknown. By analogy to models of cargo selection in clathrin-coated vesicles, it has been proposed that the vesicle coat proteins may serve as cargo receptors and concentrate cargo for packaging into vesicles (Schekman and Orci, 1996). It is tempting to speculate that the patches of Sec16p on the ER membrane act to concentrate cargo molecules at the site of future vesicle formation (Fig. 2). By functioning as a vesicle bud site selection molecule, Sec16p would couple the processes of cargo selection and vesicle coat assembly. In a simple extension of this model, the other COPII coat proteins could also bind to cargo molecules and recruit them to the site of vesicle formation. The identification of Chs1p and Hkr1p as potential binding partners for Sec16p is consistent with this hypothesis. Interestingly, six of the eight proteins identified by two-hybrid screening with Sec16p, including Hkr1p, also show a two-hybrid interaction with Sec23p. High resolution immunoelectron microscopy studies analyzing the colocalization of Sec16p and cargo proteins are in progress to test these hypotheses.

The unusual biochemical properties of Sec16p, namely the ability to form a stable oligomer and to tightly associate with the ER membrane, distinguish Sec16p from the other COPII proteins. These data suggest that Sec16p organizes the assembly of the COPII proteins and possibly specifies the site of vesicle formation. The assembly of the COPII vesicle coat may be viewed to occur in the reverse order to that of clathrin-coated vesicle assembly (Schmid, 1997). Sec16p (clathrin) can self-assemble and may form a large structure on the membrane. COPII proteins (AP complexes) might then be recruited to this structure and assist in the sorting and concentration of cargo proteins. Polymerization of the COPII subunits ultimately results in vesicle budding and transport of proteins to the Golgi. Clearly, we are just beginning to understand

the interactions involved in the formation of ER to Golgi transport vesicles. The models presented here are highly speculative, but are intended to illustrate and to outline some of the major questions that remain to be addressed.

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Figure 1. Model for the assembly of the COPII proteins on a lattice of Sec16p molecules. Oligomerization of Sec16p may lead to the formation of a lattice that acts to specify the site of vesicle bud formation

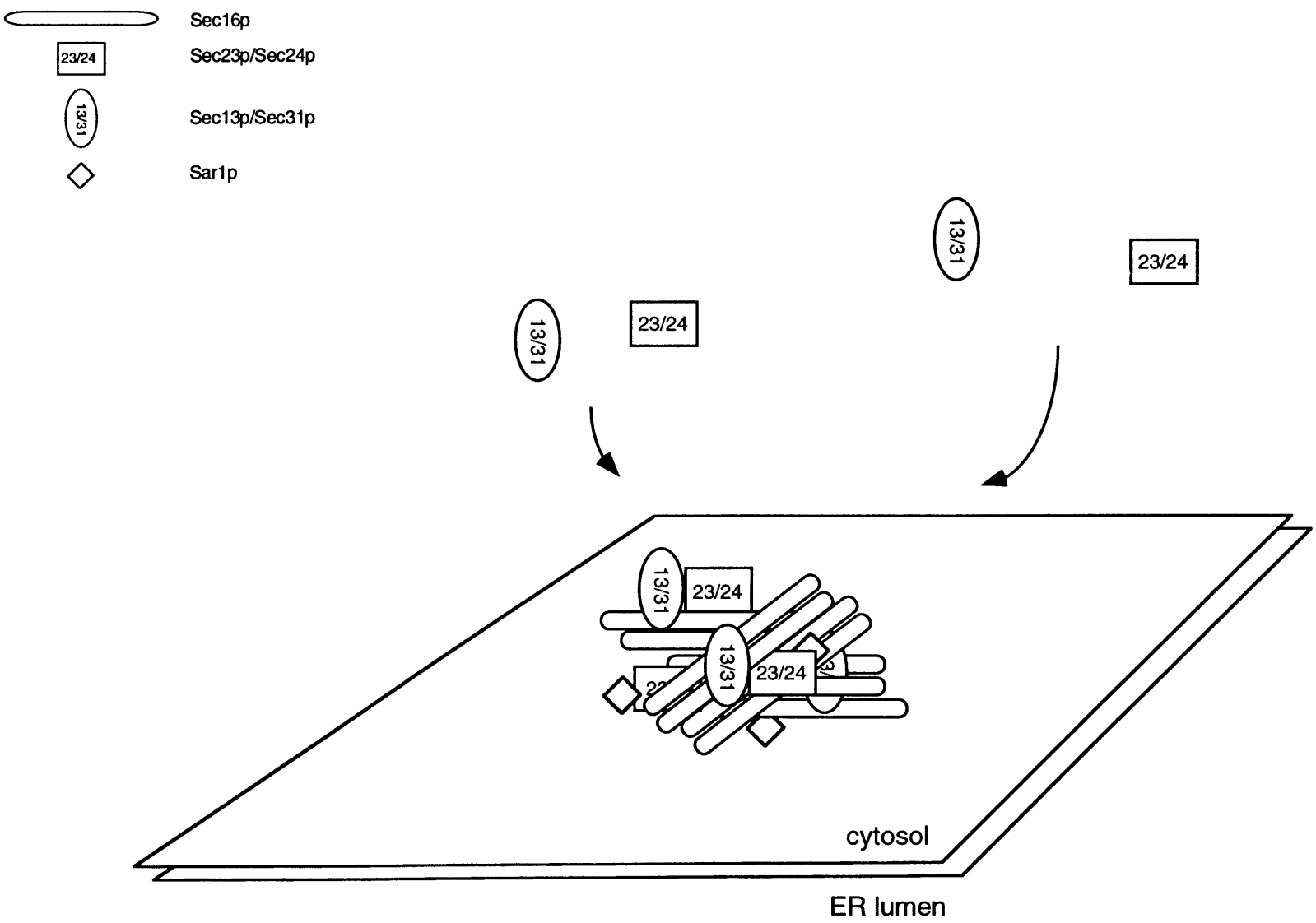
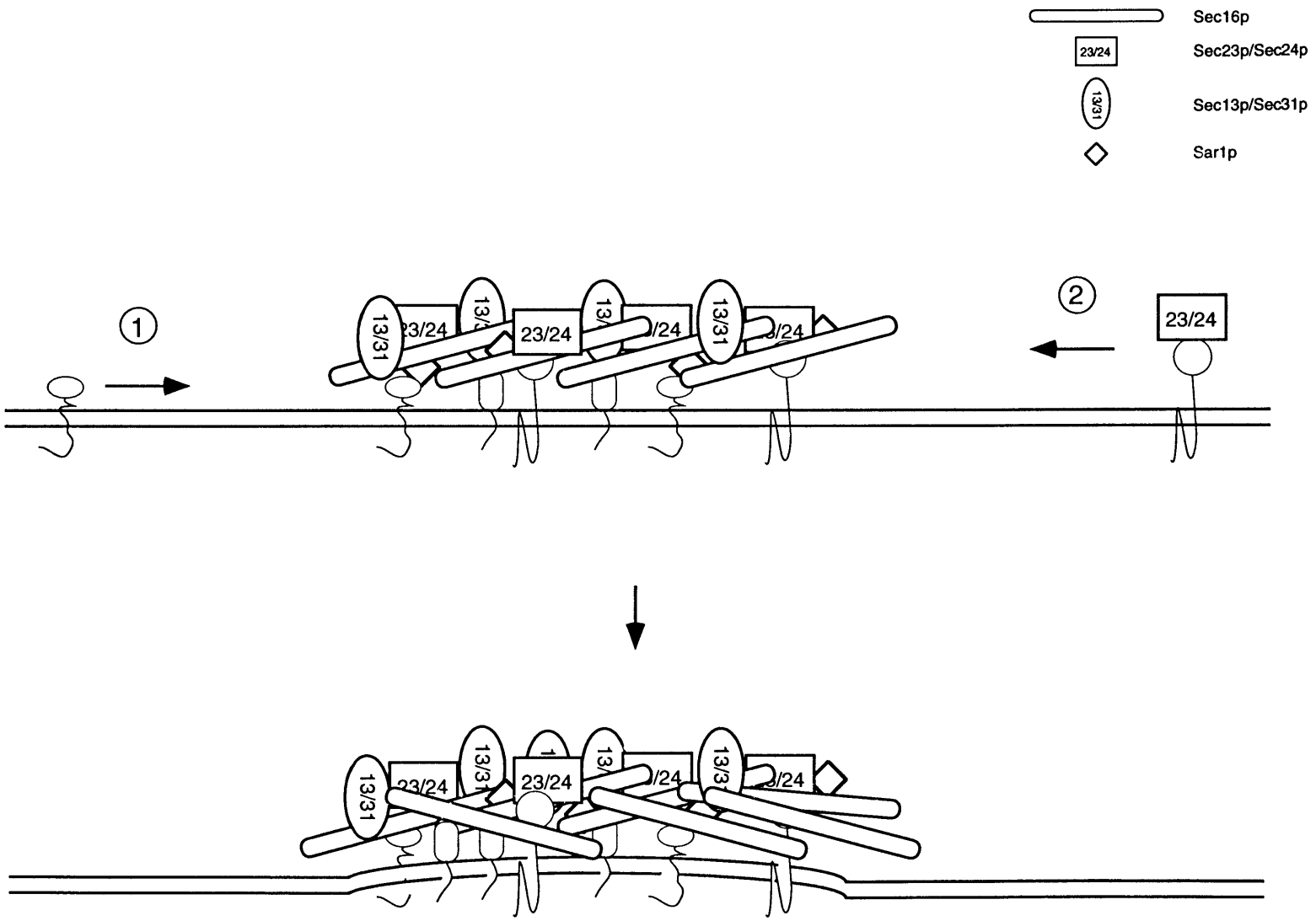

Sec16p
23/24
Sec23p/Sec24p
13/31
Sec13p/Sec31p
Sar1p
13/31
23/24
13/31
23/24
13/31
23/24
13/31
23/24
cytosol
ER lumen

Figure 2. Model for the packaging of cargo into COPII coated vesicles at the ER. Cargo molecules may be recruited to the vesicle bud site through an interaction with pre-assembled COPII components (1) or escorted through an association with COPII proteins (2).


Sec16p
23/24
Sec23p/Sec24p
13/31
Sec13p/Sec31p
Sar1p
1
2
23/24
13/31

Additional Publications

Gimeno, R.E., P. Espenshade, and C.A. Kaiser. 1995. *SED4* encodes a yeast ER protein that binds Sec16p and participates in vesicle formation. *J. Cell Biol.* 131:325-338.

Abstract

SEC16 is required for transport vesicle budding from the ER in *Saccharomyces cerevisiae*, and encodes a large hydrophilic protein found on the ER membrane and as part of the coat of transport vesicles. In a screen to find functionally related genes, we isolated *SED4* as a dosage-dependent suppressor of temperature-sensitive *SEC16* mutations. Sed4p is an integral ER membrane protein whose cytosolic domain binds to the C-terminal domain of Sec16p as shown by two-hybrid assay and coprecipitation. The interaction between Sed4p and Sec16p probably occurs before budding is complete, because Sed4p is not found in budded vesicles. Deletion of *SED4* decreases the rate of ER to Golgi transport, and exacerbates mutations defective in vesicle formation, but not those that affect later steps in the secretory pathway. Thus, Sed4p is important, but not necessary, for vesicle formation at the ER.

Sec12p, a close homologue of Sed4p, also acts early in the assembly of transport vesicles. However, *SEC12* performs a different function than *SED4* since Sec12p does not bind Sec16p, and genetic tests show that *SEC12* and *SED4* are not functionally interchangeable.

The importance of Sed4p for vesicle formation is underlined by the isolation of a phenotypically silent mutation, *sar1-5*, that produces a strong ER to Golgi transport defect when combined with *sed4* mutations. Extensive genetic interactions between *SAR1*, *SED4* and *SEC16* show close functional links between these proteins and imply that they might function together as a multisubunit complex on the ER membrane.

Shaywitz, D.A., P. Espenshade, R.E. Gimeno, and C.A. Kaiser. 1997. COPII subunit interactions in the assembly of the vesicle coat. *J. Biol. Chem.* 272:25413-25416.

Abstract

In vitro analysis of COPII vesicle formation in the yeast *S. cerevisiae* has demonstrated the requirement for three cytosolic factors: Sec31p/Sec13p, Sec23p/Sec24p, and Sar1p. Convergent evidence suggests that the peripheral endoplasmic reticulum (ER) membrane protein Sec16p also represents an important component of the vesicle assembly apparatus: *SEC16* interacts genetically with all five COPII genes; Sec16p binds to Sec23p and Sec24p, is found on ER-derived transport vesicles, and is required in vitro for the efficient release of ER-derived cargo. In this report, we demonstrate an important functional interaction between Sec16p and Sec31p. First, we map onto Sec31p binding regions for Sec16p, Sec23p, Sec24p, and Sec13p. Second, we show that a truncation mutant of Sec31p specifically defective for Sec16p binding is unable to complement a *sec31Δ* mutant and cannot rescue the secretion defect of a temperature-sensitive *sec31* mutant at non-permissive temperatures. We propose that Sec16p organizes the assembly of a coat which is stabilized both by the interaction of Sec31p with Sec23p and Sec24p, as well as by the interaction of all three of these components with Sec16p.

Roberg, K.J., P. Espenshade, R.E. Gimeno, and C.A. Kaiser. 1997. Submitted.

Abstract

In *Saccharomyces cerevisiae*, temperature-sensitive alleles of *sec12*, *sec13*, *sec16*, and *sec23* block COPII vesicle formation at nonpermissive temperatures, but affect neither growth nor secretion at 24°C. In contrast, most double-mutant combinations of these alleles are lethal even at 24°C. To identify novel genes involved in COPII vesicle formation, we screened for mutations lethal in combination with *sec13* at 24°C. We identified nine genes that we designated *LST* (Lethal with sec-thirteen). Of these, only *lst1* and *lst6* showed genetic interactions with other *sec* genes involved in vesicle formation at the ER. We have found that Lst1p is a homologue of the COPII subunit Sec24p. Like Sec24p, Lst1p is a peripheral ER-membrane protein that can form a complex with the COPII subunit Sec23p. Although *lst1Δ* mutants are viable at 24°C on all media, they grow poorly at higher temperatures on acidic media. This sensitivity to low pH results from a specific defect in the export of the plasma membrane proton-ATPase from the ER. We propose that a modified COPII coat, with Lst1p in place of Sec24p, is required for the efficient export of Pma1p from the ER.