Proteins that Interact with Sec16p during COPII Vesicle Formation in *Saccharomyces cerevisiae*

by Ruth E. Gimeno

Submitted to the Department of Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at the
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Signature of Author_____
Department of Biology
September 1996

Certified by_____
Chris A. Kaiser
Thesis Supervisor

Accepted by_____
Rick Young
Chairperson, Departmental Committee on Graduate Studies

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Abstract

COPII coated vesicles transport secretory proteins from the endoplasmic reticulum (ER) to the Golgi apparatus. Six proteins are required for COPII vesicle formation and are part of the COPII coat. Five of these proteins - two heteromeric complexes, Sec23p/Sec24p and Sec13p/Sec31p, and a small GTPase, Sar1p - are present in the cytosol. The sixth protein, Sec16p, is tightly associated with the ER membrane.

The function of Sec16p was explored by investigating the role of proteins that interact with Sec16p. Two cytosolic COPII coat components, Sec23p and Sec24p, bind independently to distinct regions of Sec16p. Sec23p binds to the C-terminal domain of Sec16p, while Sec24p binds to the central domain of Sec16p. These interactions suggest that Sec16p may act as a scaffold for the assembly of soluble coat proteins.

An integral ER membrane protein, Sed4p, was identified in a screen for dosage-dependent suppressors of temperature-sensitive *sec16* mutants. The cytosolic domain of Sed4p binds to the C-terminal domain of Sec16p. Sed4p is likely to interact with Sec16p before vesicle budding is complete, since Sed4p is present in the ER membrane, but not on vesicles. Deletion of *SED4* decreases the rate of ER to Golgi transport and exacerbates mutants defective in vesicle formation, suggesting that Sed4p is important, but not necessary for vesicle formation at the ER. Extensive genetic interactions between *SED4*, *SAR1* and *SEC16* show close functional links between these proteins and imply that they might function together as a multi-subunit complex on the ER membrane.

Sec12p, a close homolog of Sed4p, also acts early in the assembly of transport vesicles. However, *SEC12* performs a different function from *SED4*

because Sec12p does not bind Sec16p, and genetic tests show that *SEC12* and *SED4* are not functionally interchangeable.

Iss1p, a close homolog of Sec24p, was identified in a two-hybrid screen for proteins that bind to the central domain of Sec16p. Deletion of *iss1* and a temperature-sensitive *sec24* mutant exacerbate mutants defective in vesicle docking, suggesting that Iss1p and Sec24p function to facilitate docking of ER-derived vesicles.

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

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Chapter 1

Coat-Mediated Vesicle Formation

PREFACE

Part of this chapter has been submitted for publication in "The Molecular and Cellular Biology of the Yeast Saccharomyces". Vol. 3. Broach, J. R., Pringle, J., and E. Jones Eds. Cold Spring Harbor Laboratory Press, as:

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OVERVIEW

The secretory pathway of eukaryotic cells consists of a series of intracellular, membrane-bounded compartments. Transport between the compartments of the secretory pathway occurs by vesicles that form by budding from one compartment and are consumed by fusion with the next (Palade, 1975). In each compartment, proteins to be secreted have to be sorted from resident proteins, and secreted proteins have to be targeted to the correct acceptor compartment. An important goal in understanding the secretory pathway is to understand how vesicles are formed at different steps in this pathway and how this process achieves sorting of cargo molecules. Since docking molecules that recognize the acceptor membrane are components of the vesicle, vesicle formation is also likely to affect targeting to the acceptor membrane.

In the most well studied cases, vesicle formation is associated with the formation of a coat of peripheral membrane proteins around the budding vesicle. Three distinct coats -- the clathrin coat, the coatomer coat and the COPII coat -- have been identified. Although the subunit composition of each coat is different, the three coats are similar in the use of a small GTP-binding protein to regulate the association of cytosolic coat components with the membrane. Furthermore, in all three coats vesicle formation is coupled to the sorting of cargo molecules. Weak sequence homology between components of the coatomer and the clathrin coat suggests that these two coats are distantly related (Cosson et al., 1996).

Formation of clathrin and coatomer coated vesicles has been analyzed extensively in vitro; these studies suggest that coat-mediated vesicle formation involves at least three biochemically distinct steps. First, cytosolic coat components assemble onto the membrane. In both clathrin and coatomer coats, this process is coupled to the sorting of cargo proteins. Second, the membrane has to curve to form a vesicle bud of defined diameter. Finally, the curved

membrane has to pinch off to form a coated vesicle. For each coat it will be important to understand how these steps occur and how they are regulated.

Since both the clathrin and the coatomer coat have been used as a paradigm for vesicle formation mediated by the COPII coat, I will review briefly the literature concerning these two coats before discussing in detail what is known about the COPII coat.

CLATHRIN COATED VESICLES

The clathrin coat consists of two heteromeric protein complexes, a complex containing clathrin light and heavy chain ("clathrin"), and a tetrameric complex, termed adaptor protein complex (AP complex). Clathrin coated vesicles mediate transport from the trans Golgi compartment to the lysosome and endocytic uptake at the plasma membrane; two distinct, though homologous, AP complexes, AP-1 (trans Golgi) and AP-2 (plasma membrane), are used for these two processes. Both AP-1 and AP-2 complexes bind to the cytosolic domain of integral membrane cargo proteins through a conserved motif characterized by aromatic amino acid residues (Robinson, 1994).

The first step in clathrin coated vesicle formation is the recruitment of AP complexes to the membrane. This assembly reaction is mediated by at least two classes of proteins, the small GTPase ARF and cargo proteins. ARF was first described as a component of coatomer coated vesicles and has been studied primarily in this context; the properties of ARF will be discussed in detail in the coatomer section (see below). A role for ARF in AP-1 recruitment was suggested by the observation that conditions that remove ARF from membranes inhibit AP-1 association with Golgi membranes in vivo (Robinson and Kreis, 1992). In vitro assays that measure binding of AP-1 complexes to purified Golgi membranes or to permeabilized cells demonstrated unequivocally that ARF is required for high

affinity binding to Golgi membranes (Traub et al., 1993; Stamnes and Rothman, 1993; Le Borgne et al., 1996). However, ARF is not sufficient for high affinity AP-1 binding: conditions that increase the amount of ARF bound to Golgi membranes in vitro do not increase the number of high affinity AP-1 binding sites (Le Borgne et al., 1996). Thus, ARF may be one component of a multi-subunit AP-1 receptor or it could act by activating downstream elements for AP-binding (see coatomer section below).

The second class of proteins implicated in AP recruitment are integral membrane cargo proteins. A recent study by Le Borgne et al. (1996) shows that the number of high affinity AP-1 binding sites on Golgi membranes correlates with the amount of cargo proteins present, strongly suggesting that cargo proteins are part of the AP-1 binding site. However, cargo molecules are not sufficient for AP-1 recruitment to membranes, as demonstrated by the inability of membranes to recruit AP-1 in the absence of ARF.

Once AP complexes are bound, clathrin has to be recruited to AP-containing membranes. Since clathrin and purified AP complexes bind to each other in vitro, clathrin may bind to AP complexes directly (Pearse and Robinson, 1984; Smythe et al., 1992; Zaremba and Keen, 1993).

An interesting paradox is presented by the observation that both clathrin and adaptor complexes are present in the cytosol at concentrations that are high enough to allow binding to each other in vitro, but the two complexes do not appear to associate in the cytosol in vivo (Smythe et al., 1992; Zaremba and Keen, 1993). It has been suggested that cytosolic clathrin is bound to inhibitors, such as valosin-containing protein and Hsc70, that block association with adaptins (Robinson, 1994); presumably, adaptins bound to cargo containing membranes are able to remove these inhibitors from clathrin. Alternatively,

additional molecules on the membrane may act together with adaptor complexes to recruit clathrin from the cytosol.

Clathrin associated with membranes forms two distinct structures: flat sheets and curved lattices (Heuser, 1980; Maupin and Pollard, 1983; Miller et al., 1991). Experiments using cells depleted of potassium suggest that clathrin lattices first form as planar sheets and then proceed to form curved lattices (Larkin et al., 1986). When the number of cargo proteins for clathrin coated vesicles on the plasma membrane is increased, the amount of flat clathrin sheets increases accordingly, demonstrating that formation of flat clathrin lattices is mediated by cargo proteins (Miller et al., 1991). Since the amount of clathrin coated pits does not increase with the number of cargo proteins, it has been suggested that the transition from flat to curved clathrin lattices is regulated and requires factors in addition to cargo proteins (Miller et al., 1991). At present the biochemical mechanism(s) for the transition from flat to curved clathrin lattices are unknown.

After formation of a clathrin coated bud, the bud becomes constricted, and then pinches off the membrane. A biochemical assay has been developed that detects formation of a constricted bud by measuring the accessibility of plasma membrane proteins to either antibodies or small molecules (Schmid and Smythe, 1991). Experiments using this assay demonstrate that bud formation, constriction and pinching off are distinct reactions (Schmid and Smythe, 1991; Carter et al., 1993). In particular, formation of constricted coated pits requires GTP, but not GTP hydrolysis, while pinching off requires GTP-hydrolysis (Carter et al., 1993).

A 100 kD GTP-binding protein, dynamin, has been implicated in constricting the bud and then allowing scission to occur. Mutants in dynamin block budding of clathrin coated vesicles, but allow the formation of coated pits

(Kosaka and Ikeda, 1983; Damke et al., 1994). Interestingly, in GTPγS treated nerve terminals, tubular invaginations accumulate that are similar to the ones seen in dynamin mutants; the stalks of these invaginations are surrounded by rings containing dynamin, and the invaginations are often capped by a clathrin coated bud (Takel et al., 1995). This study demonstrates that dynamin can localize to the neck of clathrin coated buds, a position that would be expected for a protein that mediates constriction and scission. Since dynamin self-assembles into rings and stacks of interconnected rings in vitro, it has been suggested that dynamin assembly is directly responsible for constricting the neck of clathrin coated vesicles (Hinshaw and Schmid, 1995). In a model for bud constriction and scission, GDP to GTP exchange on dynamin allows assembly of dynamin rings around the neck of clathrin coated buds, thus constricting them at the neck. GTP hydrolysis on dynamin is then thought to induce a conformational change that closes the rings, resulting in a budded vesicle (Hinshaw and Schmid, 1995).

COATOMER COATED VESICLES

Coatomer is a seven subunit protein complex that is present in the cytosol and forms a coat on Golgi-derived vesicles (Waters et al., 1991; Serafini et al., 1991; Rothman, 1994). Coatomer-coated vesicles mediate retrograde transport from the Golgi apparatus to the ER (Letourneur et al., 1994), and have also been suggested to participate in transport from the ER to the Golgi apparatus (Bednarek et al., 1995; Aridor et al., 1995) and in transport between Golgi cisternae (Orci et al., 1986; Rothman, 1994). The role of coatomer in the last two process, however, is disputed (see Pelham, 1994 for review).

Coatomer recruitment to the membrane probably involves two classes of proteins: the small GTP-binding protein ARF and cargo molecules. ARF is a 21 kD myristoylated protein that is absolutely required for coatomer binding to Golgi

membranes from chinese hamster ovary cells. Vesicles made from these membranes in vitro contain stoichiometric amounts of ARF and coatomer, suggesting that ARF is a component of the coatomer coat (Orci et al., 1993; Ostermann et al., 1993). ARF binding to membranes precedes and modulates coatomer binding suggesting that ARF recruits coatomer to the membrane (Donaldson et al., 1992a; Palmer et al., 1993; Ostermann et al., 1993).

The mechanism by which ARF associates specifically with Golgi membranes has been the subject of considerable interest. Membrane binding of ARF is regulated by its GTPase cycle: ARF in its GTP bound form is stably bound to membranes, while ARF-GDP is present in the cytosol (Walker et al., 1992; Palmer et al., 1993; Donaldson and Klausner, 1994). Since ARF-GTP can bind to phospholipids and phospholipid vesicles (Kahn, 1991; Walker et al., 1992), it has been suggested that the association of ARF-GTP with membranes is due to the intrinsic affinity of ARF-GTP for lipids. Nucleotide exchange factor(s) for ARF in the Golgi membrane (Donaldson et al., 1992b; Helms and Rothman, 1992) may direct the specific association of ARF with these membranes in vivo. In addition to non-specific binding, a specific ARF receptor on Golgi membranes has been postulated, based on the observation that ARF is bound to membranes in two distinct pools: a liposome extractable, non-saturable pool and a non-extractable, saturable pool. It has been suggested that ARF-GTP is first non-specifically bound to the membrane and then recruited to its receptor where it forms a stable interaction (Helms et al., 1993).

The role of ARF in coatomer recruitment has recently been reexamined, after it was found that ARF activates phospholipase D (PLD; Brown et al., 1993; Cockcroft et al., 1994). Recent data suggest that the effects of ARF on coatomer assembly could be mediated by its ability to stimulate phospholipase D (PLD), implying that ARF is not an obligatory component of the coatomer coat (Ktistakis

et al., 1995; Ktistakis et al., 1996). Thus, Golgi membranes from cell lines with high intrinsic PLD activity do not require ARF for budding of coatomer coated vesicles and the vesicles that form do not contain stoichiometric amounts of ARF (Ktistakis et al., 1995; Ktistakis et al., 1996). Furthermore, membranes from chinese hamster ovary cells that have been preincubated with ARF and reisolated are capable of forming coatomer coated vesicles in the apparent absence of ARF (Ktistakis et al., 1995). Phosphatidic acid, a reaction product of PLD, stimulates coatomer binding to liposomes and has been suggested to mediate the effects of ARF on coatomer binding (Ktistakis et al., 1996).

Purified coatomer binds to the cytosolic domain of cargo proteins in vitro; the ER recycling motif KKXX appears to be a critical component of the coatomer binding site (Cosson and Letourneur, 1994). This observation suggests that the cytosolic coatomer complex could be recruited to the membrane by cargo proteins in a manner similar to what has been suggested for clathrin coat components. Thus far, however, the role of cargo proteins in coatomer recruitment has not been examined directly.

Little is known about events that lead to budding after recruitment of the coatomer coat. For instance, it is unclear whether the coatomer complex can assemble into distinct flat and curved state similar to clathrin lattices. Furthermore, it is unknown how the coatomer coated bud is constricted and how it pinches off. Since coatomer coated buds formed in vitro with purified components are unable to proceed to form vesicles (Orci et al, 1983; Ostermann et al., 1993), distinct requirements for vesicle budding must exist. In vitro, these requirements can be met by addition of fatty acyl-coenzyme A (Ostermann et al., 1993); however, it is unclear, whether fatty acyl-coenzyme A participates in vesicle budding in vivo.

COPII COATED VESICLES

COPII coated vesicles are the primary vehicle for transporting secretory proteins from the ER to the Golgi apparatus in both the yeast *Saccharomyces cerevisiae* and, probably, in mammalian cells.

Protein Transport from the Endoplasmic Reticulum to the Golgi Apparatus in *Saccharomyces cerevisiae*

The dissection of ER to Golgi transport in yeast has been facilitated by two events: the discovery of conditional mutants that are defective in either vesicle formation or vesicle fusion, and in vitro reconstitution studies which have helped to define the essential components of the assembly and fusion machinery.

The first genetic screen for secretory (*sec*) mutants identified twenty-three complementation groups; analysis of the maturation of a marker protein revealed that a subset of the *sec* mutants were defective in ER to Golgi transport (Novick et al., 1981; Fig. 1). Careful morphological analysis further divided the ER to Golgi mutants into two classes: mutants required for the formation of vesicles and mutants required for the consumption of vesicles. Vesicle consumption mutants (including *sec17*, *sec18*, and *sec22*) accumulated 50nm transport vesicles, while vesicle formation mutants (including *sec12*, *sec13*, *sec16*, and *sec23*) did not (Kaiser and Schekman, 1990). Epistasis analysis confirmed that vesicle formation mutants function at an earlier stage than vesicle consumption mutants (Kaiser and Schekman, 1990).

The second approach to dissection of ER to Golgi transport is based on the in vitro reconstitution of this transport step (Baker et al., 1988; Ruohola et al., 1988). In its simplest form, in vitro ER to Golgi transport assays measure addition of Golgi-specific carbohydrate modifications to radiolabeled α-factor that has been posttranslationally translocated into the ER. Gently-lysed cells or

partially purified ER membranes can serve as a donor compartment, while the acceptor compartment is generally supplied by a membrane fraction enriched in Golgi membrane by differential centrifugation. In the presence of exogenously added cytosolic proteins and ATP, typically 25 to 50% of the input α-factor is modified in this system.

The basic in vitro assay has been modified to allow vesicle formation to be evaluated independently of vesicle fusion. The assay for vesicle budding begins with α-factor within rapidly sedimenting ER membranes, and follows its conversion to a slowly sedimenting fraction that corresponds to free vesicles (Groesch et al., 1990; Rexach et al., 1991; Rexach et al., 1994; Barlowe et al., 1994; Oka and Nakano, 1994). In this assay, mutants required for vesicle formation in vivo (such as *sec12* and *sec23*) were also found to be defective for vesicle budding in vitro. Similarly, the vesicle accumulating mutant *sec18* was shown to be defective for vesicle fusion in vitro (Rexach et al., 1991; Rexach et al., 1994; Oka and Nakano, 1994). Importantly, these studies of mutants establish a faithful correspondence between the in vitro transport assay and the events that occur in vivo.

Attempts to purify ER to Golgi transport vesicles from whole cells have failed probably because of the low abundance of these organelles. Pure ER to Golgi transport vesicles can be produced in quantity by in vitro budding reactions under conditions where vesicle fusion with an acceptor compartment is inhibited by either mutation or antibody (Groesch et al., 1990; Rexach et al., 1994; Barlowe et al., 1994; Oka and Nakano, 1994). Purified ER-derived vesicles thus produced are functional transport intermediates since they are competent to fuse with Golgi membranes (Groesch et al., 1990; Rexach et al., 1994; Barlowe et al., 1994; Oka and Nakano, 1994). ER-derived vesicles produced in vitro have uniform morphology (~60 nm diameter), and when formed under the appropriate

conditions are encapsulated in a protein coat that can be visualized by electron microscopy after fixation and staining with tannic acid. This coat consists of a subset of the proteins required for vesicle formation in vivo and in vitro, and has been termed COPII (Barlowe et al., 1994). Vesicle production in vitro appears to reproduce the normal selectivity in segregation of vesicle proteins from ER proteins that occurs before or during vesicle formation, since the vesicles that bud in vitro contain known vesicle docking factors, such as Sec22p, and Bos1p, but do not contain resident ER proteins (Barlowe et al., 1994; Rexach et al., 1994). In addition, these vesicles have a characteristic set of twelve membrane proteins, termed ERV (ER-vesicle associated) proteins (Rexach et al., 1994).

Components of COPII Coated Vesicles in *Saccharomyces cerevisiae*

Vesicle formation from the ER in vitro requires both ER membranes and concentrated cytosolic protein. Five polypeptides – two heteromeric protein complexes, the Sec23p/Sec24p complex and the Sec13p/Sec31p complex, and a small GTP-binding protein, Sar1p – in pure form will satisfy this requirement for cytosolic protein in vesicle budding (Salama et al., 1993; Barlowe et al., 1994). These five proteins are present on completed vesicles and are abundant components of the COPII coat (Barlowe et al., 1994). In addition to these cytosolic factors, three other proteins -- Sec16p, Sec12p, and Sed4p -- are also involved in COPII vesicle formation. Sec16p is a protein that is tightly associated with the ER membrane and is also present on ER-derived vesicles produced in vitro (Espenshade et al., 1995). Two integral ER membrane proteins, Sec12p and Sed4p, regulate COPII vesicle formation, but are not incorporated into completed vesicles (Barlowe et al., 1994; Gimeno et al., 1995).

It is important to note that the distinction between cytosolic and membrane-associated COPII coat components is defined by the behavior of the

in vitro assay as carried out on ER membranes that have been extracted with urea to remove peripheral proteins, and may not accurately reflect the distribution of these proteins in living cells. For example, Sec23p and Sar1p have been defined as cytosolic COPII coat components in the context of the in vitro assay, yet these proteins appear to be principally associated with membranes in crude cell extracts (Hicke and Schekman, 1989; Nishikawa and Nakano, 1991).

It is also notewothy that the in vitro budding assay has been most useful for study of essential factors that can be depleted from the starting ER membranes and then be added back in soluble form. Additional peripheral membrane proteins like Sec16p that are not easily extracted from ER membranes may have escaped detection by the budding assay in its current form.

Sec23p and Sec24p: Cytosolic Vesicle Coat Proteins

SEC23 was initially isolated as an ER to Golgi mutant in the original *sec* screen, and was subsequently determined to participate in vesicle formation in vivo (Novick et al., 1981; Kaiser and Schekman, 1990). The conservation of the temperature sensitive phenotype under in vitro conditions facilitated the biochemical isolation of Sec23p activity from wild-type cytosol (Baker et al., 1988; Hicke et al., 1989). Functional Sec23p was purified as a 300-400 kD complex that contains both the 85 kD Sec23p and a 105 kD protein designated Sec24p (Hicke et al., 1992; Barlowe et al., 1994); the precise stoichiometry of these two protein partners has not yet been established. Sec24p, like Sec23p, is required for vesicle formation at the ER in vitro, as demonstrated by antibody block experiments (Hicke and Schekman, 1992). Chapter 4 of this thesis describes a temperature-sensitive *sec24* mutant; analysis of this mutant demonstrates that Sec24p is required for ER to Golgi transport in vivo. The Sec23p/Sec24p

complex represents one of the major components of the COPII vesicle coat, and is required for its formation (Barlowe et al., 1994; Bednarek et al., 1995).

Not only do Sec23p and Sec24p interact with each other, both biochemically and genetically, but they also each exhibit genetic interactions with the genes encoding the other COPII components -- *SEC13*, *SEC31*, *SEC16*, and *SAR1* (Kaiser and Schekman, 1990; Oka and Nakano, 1994; Chapter 4; Fig. 2). Work presented in Chapter 2 of this thesis shows that Sec16p binds directly to both Sec23p and Sec24p; the two protein partners bind to different sites on Sec16p, and the binding of one subunit can occur in the absence of the other. Sec23p has also been shown to function as a GTPase-activating protein (GAP) for Sar1p, stimulating its GTPase activity by a factor of ten; Sec24p does not appear to affect the GAP activity of Sec23p (Yoshihisa et al., 1993). While the GAP activity of Sec23p has also stimulated the generation of several different hypotheses regarding Sec23p function (Oka and Nakano, 1994; Schekman and Orci, 1996), the exact role of the Sec23p/Sec24p complex in the formation of COPII vesicles remains to be established.

Sec13p and Sec31p: Cytosolic Vesicle Coat Proteins

SEC13, like *SEC23*, was isolated in the original sec mutant screen, and, like *SEC23*, was shown to be required for the vesicle formation step of ER to Golgi transport (Novick et al., 1981; Kaiser and Schekman, 1990). Immunodepletion experiments revealed a requirement for Sec13p in the in vitro transport assay (Pryer et al., 1993; Salama et al, 1993). Purification of Sec13p activity from wild-type cytosol revealed that Sec13p exists as a 700 kD complex with the 150 kD Sec31p protein (Pryer et al., 1993; Salama et al, 1993); the exact stoichiometry of Sec13p and Sec31p in this complex has not yet been established. Both *SEC13* and *SEC31* are essential genes, required for vesicle

transport in vivo and in vitro (Kaiser and Schekman, 1990; Salama et al., 1993; Barlowe et al, 1994; Wuestehube et al., 1996). The Sec13p/Sec31p complex, like the Sec23p/Sec24p complex, represents a major component of the COPII coat and is required for its formation (Barlowe et al., 1994; Bednarek et al, 1995).

Both *SEC13* and *SEC31* exhibit genetic interactions with the other COPII genes (*SEC23*, *SEC24*, *SAR1*, *SEC16*) (Kaiser and Schekman, 1990; Gimeno et al, 1995; A. Frand and C. Kaiser, unpublished observations; Fig. 2). Structurally, Sec13p appears to consist almost entirely of WD-40 repeats, a motif first described in the β-subunit of trimeric G-proteins, and subsequently shown to specify a β-propeller structure (Wall et al., 1995; Neer and Smith, 1996). Sec13p appears to interact with a WD-40-containing domain of Sec31p, raising the possiblility of a homotypic interaction (Shaywitz et al., in preparation). A different region of Sec31p has recently been shown to bind Sec16p, consistent with the proposed role of Sec16p as COPII scaffold (Shaywitz et al., in preparation).

The function of the Sec13p/Sec31p complex in the formation of COPII vesicles remains incompletely understood, a conclusion emphasized by recent data which suggests that Sec13p may not always be required for ER to Golgi transport. In particular, loss of function mutations in at least three genes (*BST1*, *BST2/EMP24*, and *BST3*) have been isolated that bypass the requirement for Sec13p in ER to Golgi transport (Elrod-Erickson and Kaiser, 1996). These mutants have only subtle phenotypes on their own: mutations in *bst1-3* slow transport of a subset of secreted proteins from the ER to the Golgi apparatus and increase the rate at which ER-resident proteins, such as Kar2p, reach the Golgi apparatus (Schimmöller et al., 1995; Stamnes et al., 1995; Elrod-Erickson and Kaiser, 1996). This phenotype is consistent with a decrease in the fidelity of cargo sorting at the ER that could be explained if *BST1-3* encode components of a checkpoint that monitors fidelity of cargo sorting and prevents the budding of

immature or improperly coated vesicles (Elrod-Erickson and Kaiser, 1996). Alternatively, since Bst2p/Emp24p is a major integral membrane component of ER-derived transport vesicles, it has been suggested that Emp24p/Bst2p is a sorting receptor for a subset of cargo molecules (Schimmöller et al., 1995; see below).

Sec16p: An ER-associated Vesicle Coat Protein

SEC16 encodes an essential, multi-domain 240 kD hydrophilic protein required for ER to Golgi transport in vivo (Novick et al., 1981; Espenshade et al., 1995). Sec16p is tightly associated with the periphery of the ER, and is also found on ER-derived transport vesicles, suggesting that Sec16p is a component of the COPII coat (Espenshade et al., 1995). In contrast to the other COPII components, Sec16p cannot be extracted from membranes by urea, explaining why it does not needed to be added as a cytosolic factor to the in vitro transport assay (Espenshade et al., 1995). Work presented in Chapter 2 of this thesis shows that Sec16p binds directly to the COPII subunits Sec23p and Sec24p. More recent data suggest that Sec16p also binds to the COPII coat component Sec31p (Shaywitz et al., in preparation). Based on these protein interactions we suggested that Sec16p on the ER membrane serves as a scaffold that organizes the recruitment and assembly of COPII coat components from the cytosol (see Appendix II and Chapter 2). *SEC16* exhibits genetic interactions with the five other COPII genes, underlining the importance of Sec16p in COPII vesicle formation (Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990; Chapter 3; Fig. 2). Furthermore, multiple genetic interactions between *SEC16* and *SAR1* suggest that Sar1p could regulate the assembly of cytosolic coat components onto Sec16p (Chapter 3).

Sar1p: a GTPase Coupled to Vesicle Assembly

SAR1, an essential gene required for ER to Golgi transport, encodes a small GTP-binding protein most closely related to ARF, a GTP-binding protein that regulates assembly of the coatomer (COPI) coat (Oka et al., 1991; Barlowe et al., 1993). Sar1p is a component of the COPII vesicle coat, and is required for its formation (Barlowe et al., 1994; Bednarek et al., 1995).

SAR1 exhibits genetic interactions with the five other COPII coat components (Fig. 2). In addition, the activity of Sar1p has been shown to be modified by the COPII subunit Sec23p, which functions as a Sar1p-GAP, and by Sec12p, which functions as an exchange factor for Sar1p. Sar1p has an intrinsic guanine-nucleotide off-rate (0.07 min^{-1}) and GTPase activity (0.0011 min^{-1}) similar to those of other small GTP-binding proteins (Barlowe et al., 1993). Sec12p and Sec23p increase these rates (respectively) by about an order of magnitude and Sec12p and Sec23p together increase the rate of GTP hydrolysis by approximately 50-fold (Barlowe and Schekman, 1993; Yoshihisa et al., 1993). Like Arf1p, Sar1p requires the presence of detergents or phospholipids for GTP binding (Barlowe et al., 1993), however, unlike other small GTP-binding proteins, including ARF, Sar1p does not appear to require lipid modifications for its function (Oka et al., 1991; Barlowe et al., 1993).

In vitro studies suggest that vesicle budding requires Sar1p in its GTP-bound form (Rexach and Schekman, 1991; Oka et al., 1991; Barlowe et al. 1993; Barlowe et al., 1994; Oka and Nakano, 1994). GTP-hydrolysis is not required for vesicle formation since budding still occurs when non-hydrolyzable analogs of GTP, are used, although under these conditions greater quantities of Sec23p/Sec24p and Sec13p/Sec31p are needed (Barlowe et al., 1994). GTP-hydrolysis by Sar1p is required for the overall transport process as the vesicle

fusion step appears is sensitive to nonhydrolyzable GTP analogs (Barlowe et al., 1994; Oka and Nakano, 1994).

Sar1p is thought to function by regulating assembly and disassembly of the cytosolic COPII coat components. A simple model is that Sar1p in its GTP-bound state promotes assembly of the COPII coat, while GTP-hydrolysis by Sar1p promotes disassembly of the COPII coat (Barlowe et al., 1994). This hypothesis is supported by in vitro data demonstrating that the COPII vesicle coat produced in the presence of GTP is unstable, and readily dissociates from budded vesicles, but when non-hydrolyzable analogs of GTP are substituted in the budding reaction, the resulting COPII coat is stabilized (Barlowe et al., 1994). These in vitro studies also indicate that COPII coat disassembly occurs in two distinct steps: first, Sar1p rapidly hydrolyzes GTP and dissociates from the vesicle, leaving a coat of Sec23p/Sec24p, Sec13p/Sec31p and possibly Sec16p (Barlowe et al., 1994). Second, Sec23p/Sec24p and Sec13p/Sec31p dissociate from the vesicle (Barlowe et al., 1994); it has not been established whether the dissociation of Sec16p from the vesicle membrane ever occurs.

Sec12p and Sed4p: ER Proteins that Regulate COPII Coat Formation

Sec12p and Sed4p are integral membrane proteins that influence vesicle budding at the ER, but are not themselves incorporated into vesicles. *SEC12*, an essential gene required for the formation of ER-derived vesicles, encodes a type II transmembrane protein that localizes to the ER, but is absent from vesicles produced in vitro (Nakano et al., 1988; Nishikawa and Nakano, 1993; Barlowe et al., 1994). The cytosolic domain of Sec12p is essential and stimulates guanine-nucleotide exchange by Sar1p, whereas the luminal domain appears to be less important since truncation of this domain does not greatly interfere with *SEC12* function (d'Enfert et al., 1991a; Barlowe and Schekman, 1993). The cytosolic

domain of Sec12p may also function to recruit Sar1p to the ER membrane since overexpression of Sec12p depletes Sar1p from the cytosol and increases the membrane-associated pool of Sar1p (d'Enfert et al., 1991b; Barlowe et al., 1993). Recruitment does not appear to involve direct binding between Sec12p and Sar1p since a stable association between these proteins has not been observed. However, if Sar1p in its GTP-bound state has a high affinity for membranes, Sec12p may effect recruitment simply by stimulating guanine nucleotide exchange on Sar1p; this mechanism of membrane association would be similar to what has been suggested for ARF (see above). It appears that for Sar1p to be properly activated, the exchange activity of Sec12p must be in proximity to the ER membrane since truncations of Sec12p that liberate a soluble exchange activity inhibits vesicle budding from the ER. This inhibition can be overcome by the addition of increased amounts of Sar1p (Barlowe et al., 1993).

SED4 encodes a type II transmembrane protein that is 45% identical to Sec12p in its cytosolic and transmembrane domains, but has a divergent luminal domain (Hardwick et al., 1992); however, Sed4p does not appear to stimulate the nucleotide exchange activity of Sar1p (C. Barlowe, personal communication). The cytosolic domain of Sed4p binds to the C-terminal domain of Sec16p (Chapter 3). Since Sed4p is present on the ER, but is not incorporated into vesicles, the interaction between Sec16p and Sed4p is likely to occur before vesicle formation is complete (Chapter 3). Although the deletion of *SED4* causes only minor growth and secretion defects in wild-type cells, a strain carrying an otherwise silent *sar1-5* mutation is rendered temperature-sensitive for growth and secretion when *SED4* is deleted (Chapter 3). *SED4* exhibits genetic interactions with both *SAR1* and *SEC16*, and Sed4p has also been shown to bind to Sec16p; we therefore suggested that Sed4p together with Sec16p and Sar1p may act to initiate vesicle formation at the ER (Chapter 3).

COPII Vesicles in Mammalian Cells

Mammalian homologs of several COPII proteins have now been identified. Mammalian Sar1p, Sec13p, and Sec23p all localize to the transitional region of the ER, consistent with a role in vesicle formation at the ER (Kuge et al., 1994; Shaywitz et al., 1995; Orci et al., 1991). Furthermore, in mammalian cells, the transport of the marker protein VSV-G from the ER to the Golgi is inhibited both in vivo (by a dominant negative mutant of hamster Sar1p) and in vitro (by anti-Sar1p antibodies), suggesting that the function of Sar1p in ER to Golgi transport has been evolutionarily conserved (Kuge et al., 1994). The demonstration that reciprocal human/yeast chimeras of Sec13p both complement the secretion defect of a temperature-sensitive *sec13* mutant provides further support for the conservation of COPII component function (Shaywitz et al., 1995).

Cargo Sorting during COPII Vesicle Formation

Considerable evidence has accumulated indicating that the entry of cargo molecules into ER-derived secretory vesicles reflects an important sorting decision made by the cell. For example, although discrete retention signals (which allow resident ER proteins to be retrieved from the Golgi) have been characterized, resident ER proteins lacking these signals still exit the ER at a rate much slower than that of actual secreted proteins (Munro and Pelham, 1987; Hardwick et al., 1990). Furthermore, careful immuno-electron microscopy (EM) studies suggest that at least some secretory proteins are concentrated in both mammalian cells and yeast (Mizuno and Singer, 1993; Balch et al., 1994; Bednarek et al., 1995). Finally, COPII vesicles formed in vitro from either microsomes or from nuclear envelope preparations are enriched for proteins known to enter the secretory pathway, and seem to lack resident ER proteins (such as Kar2p, Sec12p, and Sec61p) (Barlowe et al., 1994; Bednarek et al.,

1995). These data suggest that a distinction between resident and transported protein is made in the ER, at the level of packaging into transport vesicles.

If important sorting decisions occur during the formation of COPII vesicles, it is likely that the COPII proteins themselves participate in the selection process, and communicate critical information about the selection of cargo to the budding vesicles, possibly through interactions with transmembrane proteins. Coat-mediated sorting is well documented during the formation of clathrin and COPI-coated vesicles in mammalian cells (Schekman and Orci, 1996). In these coats, sorting is mediated by binding of integral membrane cargo proteins or sorting receptors to subunits of the clathrin or the COPI coat (Ohno et al., 1995; Cosson and Letourneur, 1994). Thus far, no binding between a COPII subunit and a cargo protein has been demonstrated, although it has been suggested that Sec23p may sample cargo proteins at the ER during the initial steps of vesicle formation (Schekman and Orci, 1996).

The analysis of sorting during vesicle formation at the ER is complicated by the absence of a defined sorting signal or sorting receptor. No common signal required for exit from the ER has been defined and mutants blocked in ER to Golgi transport did not reveal a candidate sorting receptor. One attractive hypothesis is that the sorting of integral membrane proteins is mediated by the interaction of these proteins with the COPII coat. In this scheme, the coat complex would collectively function as a "receptor surface," with many potential sites available for interaction with cargo membrane proteins. The sorting of soluble cargo molecules requires additional assumptions; one possibility is that soluble cargo binds to integral plasma membrane proteins, and follows them through the secretory pathway. Alternatively, there may be a family of integral membrane proteins that function as "sorting receptors," binding on the one hand to COPII proteins, and on the other, to soluble cargo molecules. Emp24p/Bst2p

has been suggested to be such a receptor. Emp24p is an integral ER membrane protein that was cloned because it is also a prominent component of ER-derived vesicles (Schimmöller et al., 1995). A chromosomal deletion of *EMP24* is viable, but causes a kinetic defect in ER to Golgi transport of a subset of cargo proteins, suggesting a role in segregating particular cargo molecules into the vesicles. However, direct binding of Emp24p to cargo proteins has not been observed.

Emp24p/Bst2p and a second ER membrane protein, Bst1p, were also identified because their deletion allows cells to grow in the absence of Sec13p (Elrod-Erickson and Kaiser, 1996; see above). Strains deleted for Bst1p or Emp24p/Bst2p have a decreased fidelity of cargo protein sorting, evidenced by a kinetic defect in the export of a secreted protein (invertase) from the ER, and missorting of the normally ER-retained proteins Kar2p, Pdi1p, and an invertase mutant defective in signal sequence cleavage (Elrod-Erickson and Kaiser, 1996). Since *bst1* and *bst2* mutants affect both protein sorting and the requirement for a COPII coat component, Bst1p and Emp24p/Bst2p may constitute part of a checkpoint that monitors both protein sorting and COPII coat assembly, before allowing vesicle formation to proceed (Elrod-Erickson and Kaiser, 1996).

Towards a Model for COPII Vesicle Formation

Biochemical and molecular analysis of the proteins required for vesicle formation suggest an outline for the pathway of COPII coat assembly. Analogous to the known mechanism of coat recruitment in clathrin and coatomer coated vesicles, the first step in vesicle formation at the ER is thought to be guanine-nucleotide-exchange by Sar1p and recruitment of Sar1p to the ER membrane. Since Sec12p, a nucleotide exchange factor for Sar1p, increases the amount of Sar1p bound to the membrane, it is thought that Sar1p in its GTP-bound form binds to membranes either directly or through a membrane-bound receptor.

Thus far, no Sar1p receptor has been identified; in particular, no direct binding of Sar1p to either Sec12p or Sed4p has been demonstrated. However, since both Sec16p and the Sec16p-binding protein Sed4p show strong genetic interactions with Sar1p, Sed4p, possibly together with Sec16p, may serve as a receptor for Sar1p (Chapter 3).

Sar1p in its GTP-bound, membrane-associated form is thought to then recruit the cytosolic coat components Sec23p/Sec24p and Sec13p/Sec31p to the membrane. Work presented in chapter 2 of this thesis and recent results by D. Shaywitz (see above) suggest that Sec16p, a protein tightly bound to the ER membrane, acts as the receptor for these cytosolic coat components. Since the C-terminal domain of Sec16p interacts with Sed4p, a protein that is not incorporated into vesicles, but has strong genetic interactions with Sar1p, we proposed that the Sec23p/Sec24p complex binds to Sec16p first, followed by binding of Sec13p/Sec31p (Chapter 2). The proposal that Sec16p is a receptor for cytosolic coat components implies that binding of cytosolic coat components to Sec16p is regulated by Sar1p. Sar1p could affect the affinity of Sec16p for coat components directly; alternatively, proteins such as Sed4p and Sec12p could mediate this effect of Sar1p.

At present, it is unclear how cargo molecules are loaded into COPII coated vesicles. Analogous to cargo loading into clathrin and coatomer coated vesicles it might be expected that a component of the COPII coat directly binds to cargo proteins. So far, however, no binding between COPII coat components and cargo protein has been described. Sec16p would be an ideal candidate for a cargo binding protein, since it is localized at the interface between the ER membrane and cytosolic coat components; binding of cargo proteins to Sec16p could modulate its affinity for cytosolic coat components.

Little is known about the requirements for constricting COPII coated buds and pinching off vesicles. In vitro, formation of COPII coated vesicles requires only cytosolic COPII coat components and ER membranes (Barlowe et al., 1994), suggesting that no additional cytosolic factors are involved in budding. Possibly, budding factors have so far escaped detection because they are tightly associated with the ER membrane, similar to Sec16p. Alternatively, the large amounts of coat components used in the in vitro assay or the special composition of the ER membrane may allow vesicle budding to occur even in the absence of special budding factors.

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Figure 1. Genes required for ER to Golgi transport in *Saccharomyces cerevisiae*. Genes required for vesicle formation are indicated on the left, genes required for vesicle docking or fusion are indicated on the right (vesicle docking genes above the arrow, vesicle fusion genes below the arrow).

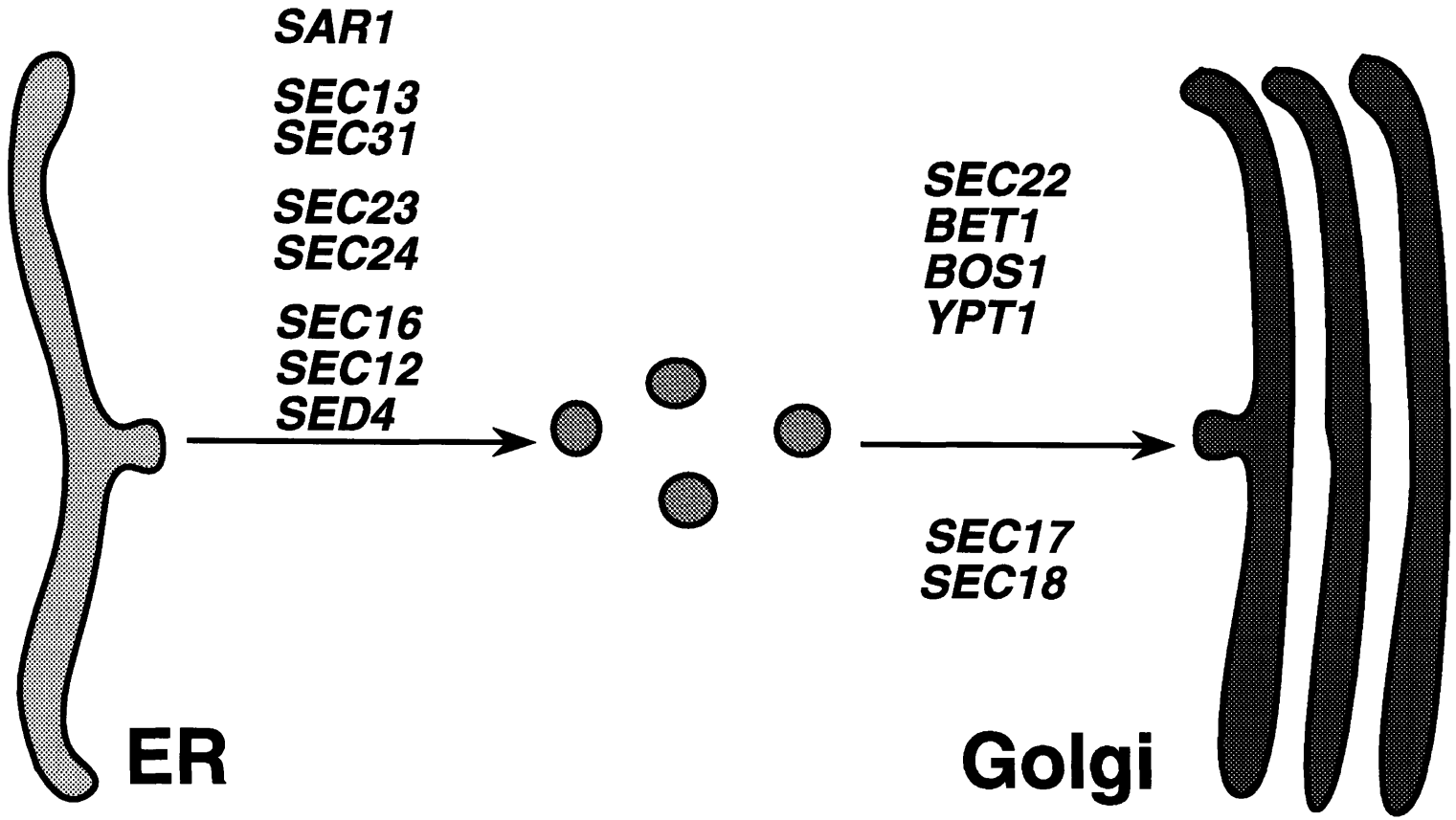
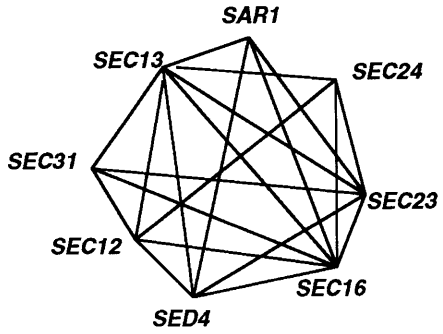

SAR1
SEC13
SEC31
SEC23
SEC24
SEC16
SEC12
SED4
SEC22
BET1
BOS1
YPT1
SEC17
SEC18
ER
Golgi

Figure 2. Interactions between ER vesicle formation genes/proteins. **A.** Synthetic lethal interactions. The following alleles were tested: *sar1-5, sec24-1, sec23-1, sec16-2, sed4-Δ1, sec12-4, sec31-1, sec13-1.* No synthetic lethal interactions were observed between *sar1-5* and *sec12-4*, and between *sec23-1* and *sec12-4*. The following combinations were not tested: *sar1-5/sec24-1, sar1-5/sec31-1, sed4-Δ1/sec24-1, sed4-Δ1/sec31-1, sec24-1/sec31-1.* **B.** Multi-copy suppression. Suppression of the following alleles was tested: *sec23-1, sec16-2, sed4-Δ1, sec12-4, sec13-1. SAR1, SED4* and *SEC12* were expressed on a high copy (2μ) plasmid, *SEC23* and *SEC16* were expressed on a low-copy (CEN) plasmid. The effect of *SEC13* overexpression has not been examined systematically. **C.** Protein-protein interactions. The interactions shown have been detected in both two-hybrid and coprecipitation experiments. No binding was detected between Sar1p and Sec16p, Sar1p and Sec12p, Sar1p and Sed4p, and between Sec12p and Sec16p. Sar1p interacted with Sec23p in the two-hybrid assay, but did not coprecipitate with Sec23p.

A


SAR1
SEC13
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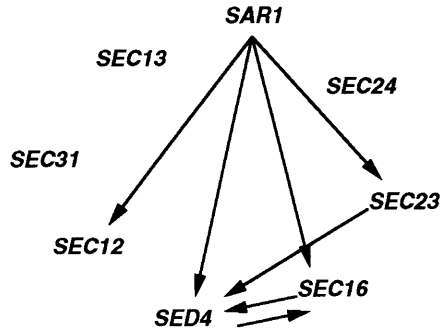
B


SAR1
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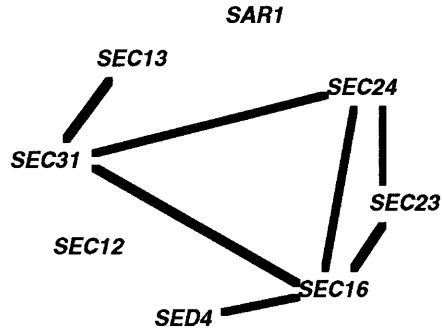
SEC12
SEC16
SED4

C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Chapter 2

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

PREFACE

This chapter represents a collaboration between myself and Peter Espenshade. Peter Espenshade purified bacterially expressed Sec23p and Sec24p and performed the in vitro binding experiments.

This chapter has been submitted in its entirety to Molecular Biology of the Cell as:
Ruth E. Gimeno, Peter Espenshade, and Chris A. Kaiser. COPII Coat Subunit Interactions: Sec24p and Sec23p Bind to Adjacent Regions of Sec16p.

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 C-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 N-terminal and C-terminal regions of Sec24p display different binding specificities. The C-terminus binds to the central domain of Sec16p while the N-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 homolog 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 C-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 C-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 N-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 N-terminus of *SEC24* was amplified by PCR and ligated into pEG202, creating pRH284. The C-terminus of *SEC24* was then inserted by ligating the 3 kb *BamH*I fragment of pAF70 into pRH284. The N-terminal *SEC24* *lexA* fusion (pRH321) contains the 2 kb *EcoR*I fragment of pRH287 ligated into pEG202. The fusion of the C-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 N-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 C-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% bromphenol 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 N-terminal 1.2 kb derived from pRH287 and the C-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 C-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 C-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 N-terminal and C-terminal portions of Sec24p (Fig. 1). Both the N-terminal and the C-terminal fusion showed a significant interaction with the central domain of Sec16p, but only the N-terminal domain interacted with Sec23p (Fig. 1 A). These tests for interaction place the Sec23p binding site in the N-terminal region of Sec24p, and suggests that both the N-terminal and the C-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 C-terminal domain of Sec24p. The C-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 C-terminal domain of Sec24p interacted only with the central domain of Sec16p (Fig. 1 B). Sec23p, in contrast, interacted only with the C-terminal domain of Sec16p but not the central domain (Fig. 1 B). These results

show that Sec23p and the C-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 N-terminal domain, and the C-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 N-terminus or the Sec24p C-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 N-terminal and C-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 N-terminus, but not fusions to the Sec24p C-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 N-terminus of Sec24p, while

binding to the central domain of Sec16p is mediated by both the N-terminus and the C-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 C-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 C-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 C-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 C-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 C-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 C-terminal region of Sec16p, whereas Sec23p only bound to the C-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 C-terminal region of Sec16p and Sec24p binds to both the central domain of Sec16p and to the C-terminal domain of Sec16p. Given that the C-terminal region of Sec24p contacts the central region of Sec16p and that the N-terminal region of Sec24p contacts Sec23p the most likely arrangement of Sec24p is with its N-terminus oriented towards the C-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 C-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 C-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 C-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 C-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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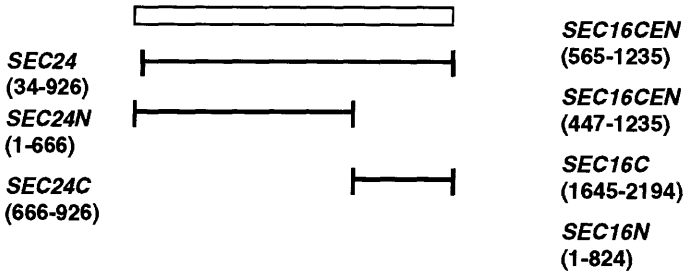
Figure 1. Two-hybrid interactions between Sec16p, Sec24p and Sec23p. The extent of each of the gene fragments of *SEC24* and *SEC16* are indicated by gene diagrams and by the amino acids numbers included in each construct given in brackets. Each interaction was evaluated by β-galactosidase assays of 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

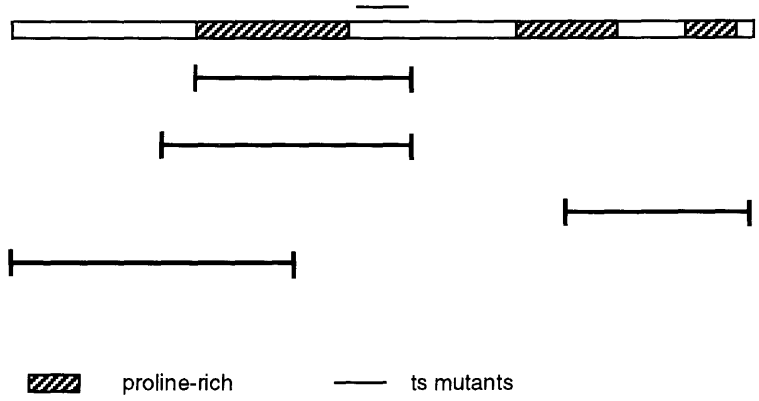


Figure 2. Affinity isolation of complexes between Sec24p, Sec16p and Sec23p from yeast extracts. The indicated fragments of Sec24p fused to GST (lanes 1-6) or GST only (lanes 7 and 8) were coexpressed with either the central domain of Sec16p fused to invertase (pRH317, upper panel) or Sec23p (pPE123, 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.

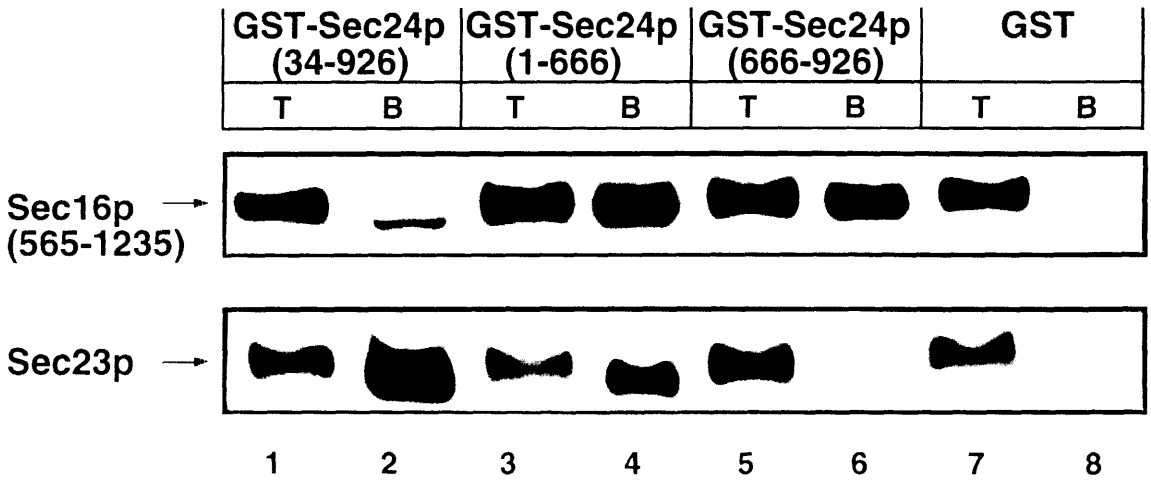

GST-Sec24p (34-926)
GST-Sec24p (1-666)
GST-Sec24p (666-926)
GST
T
B
T
B
T
B
T
B
Sec16p (565-1235)
Sec23p
1
2
3
4
5
6
7
8

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.

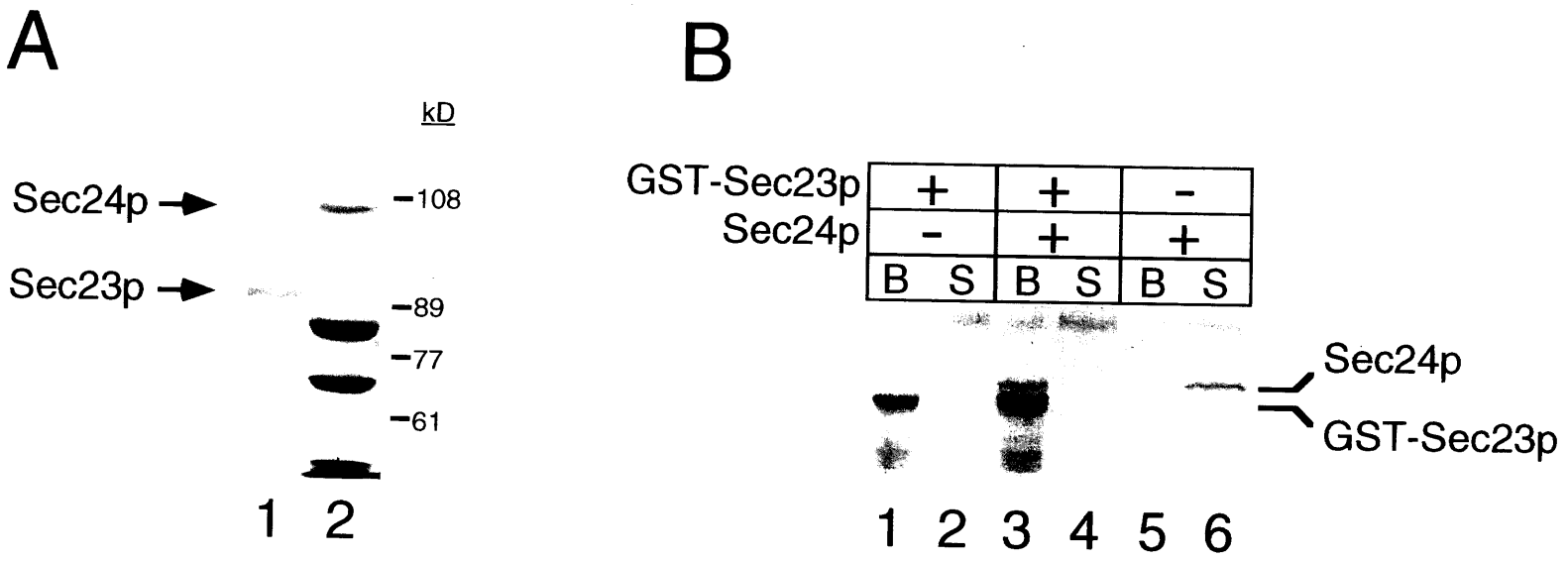
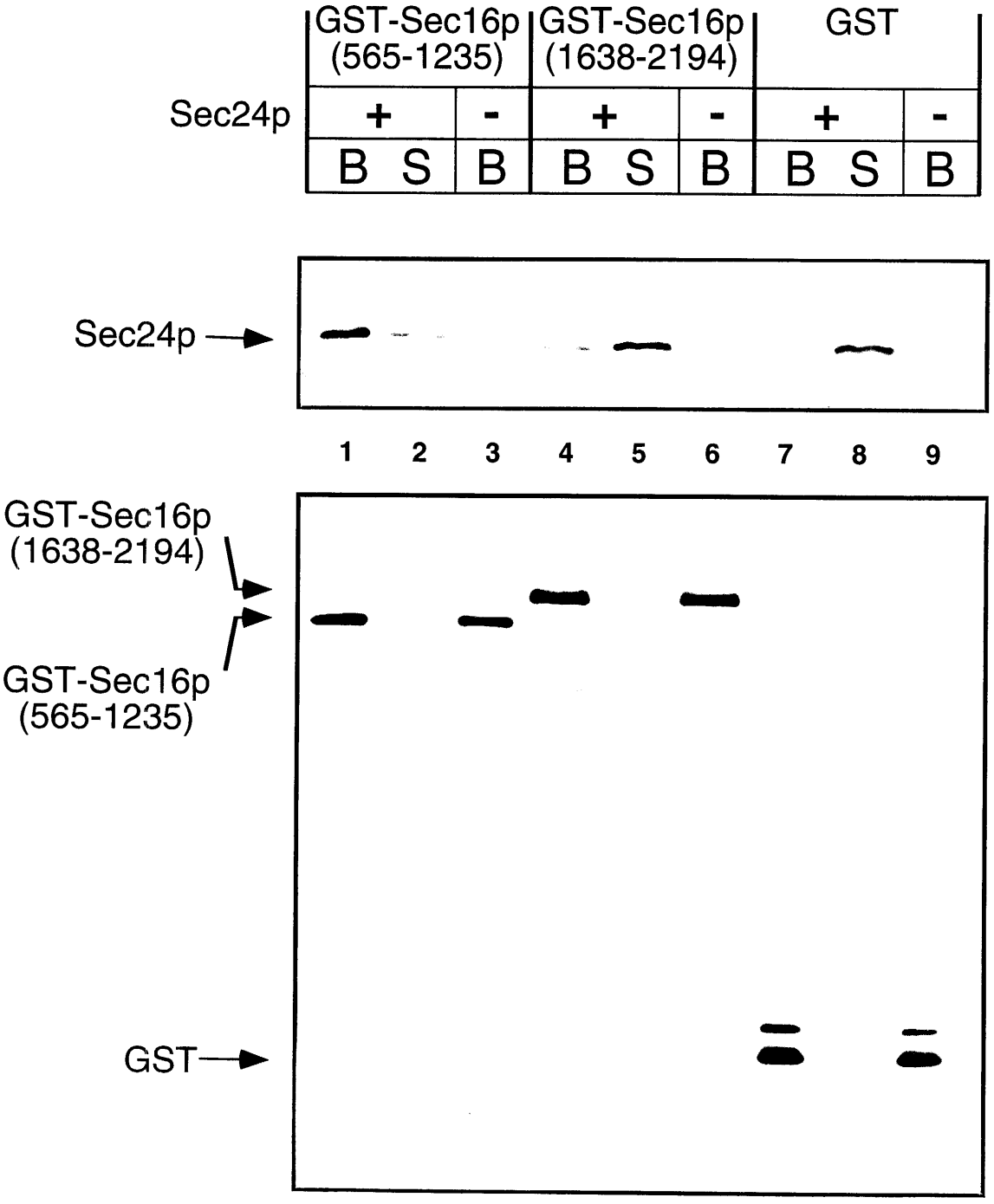
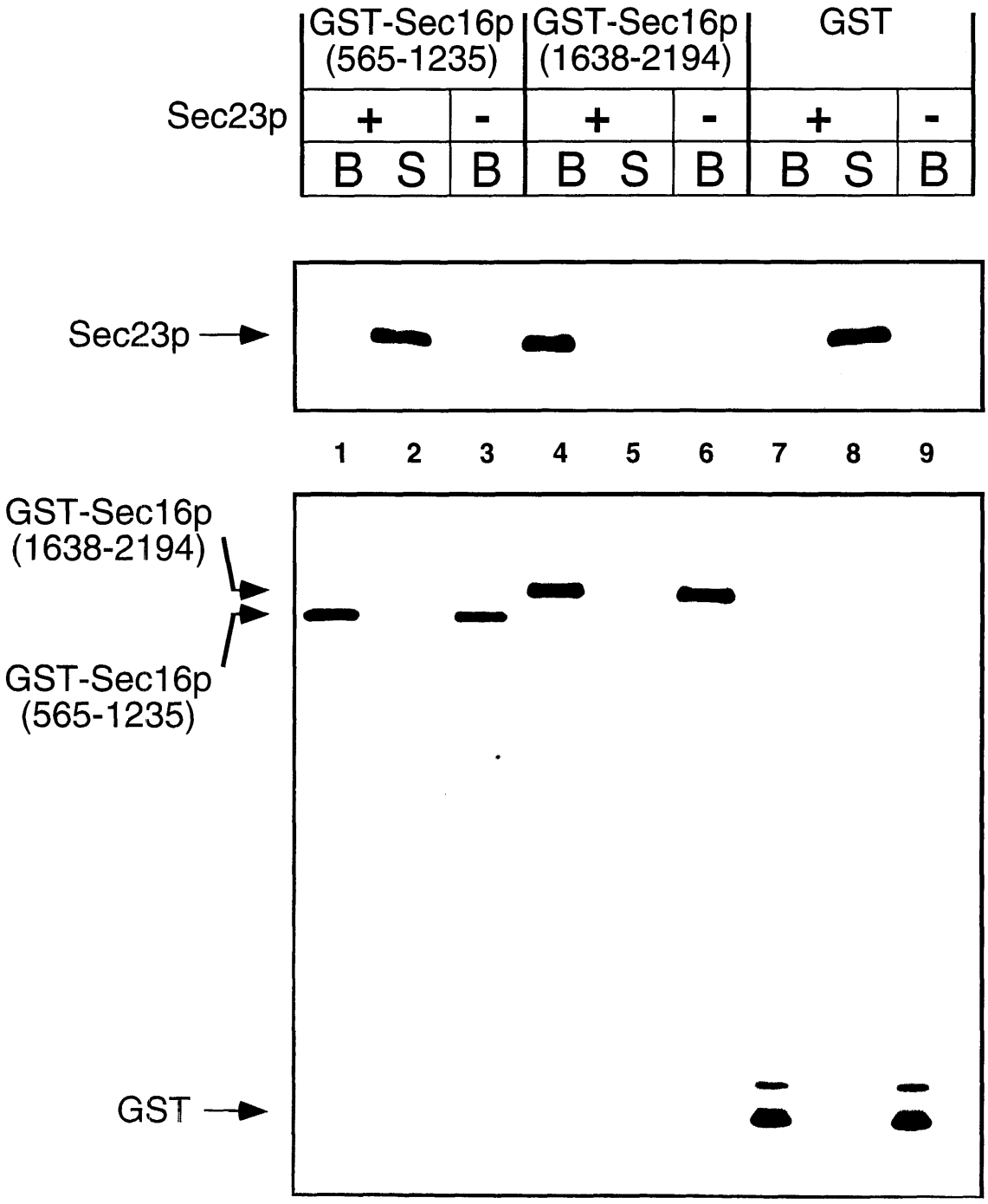

A
kD
Sec24p
Sec23p
108
89
77
61
1 2
B
GST-Sec23p
Sec24p
+ + -
- + +
B S B S B S
Sec24p
GST-Sec23p
1 2 3 4 5 6

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.

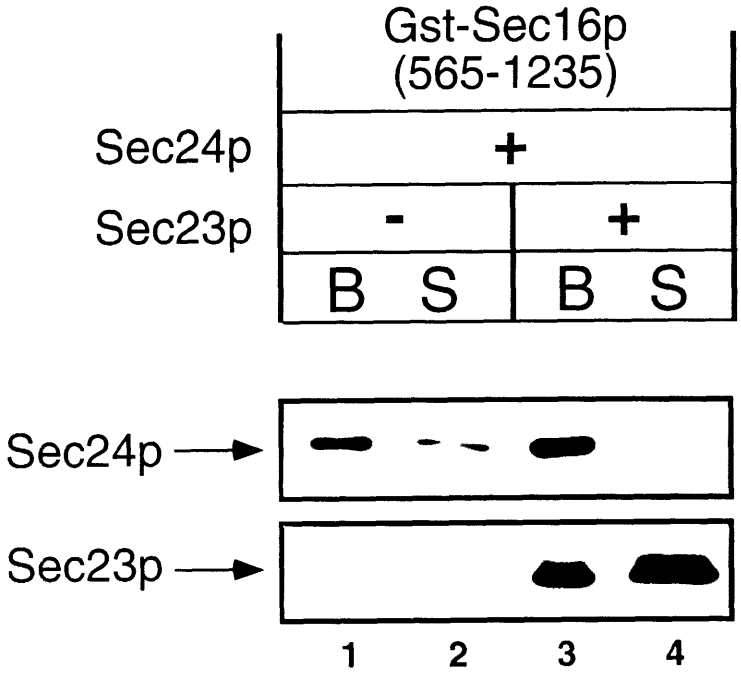
A


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

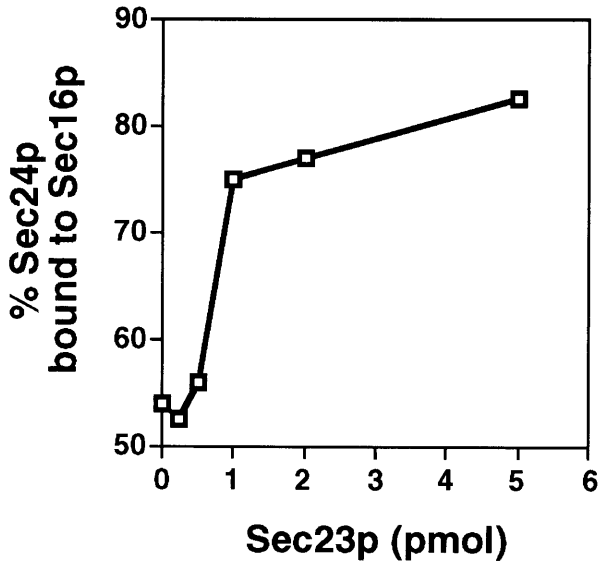
B


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

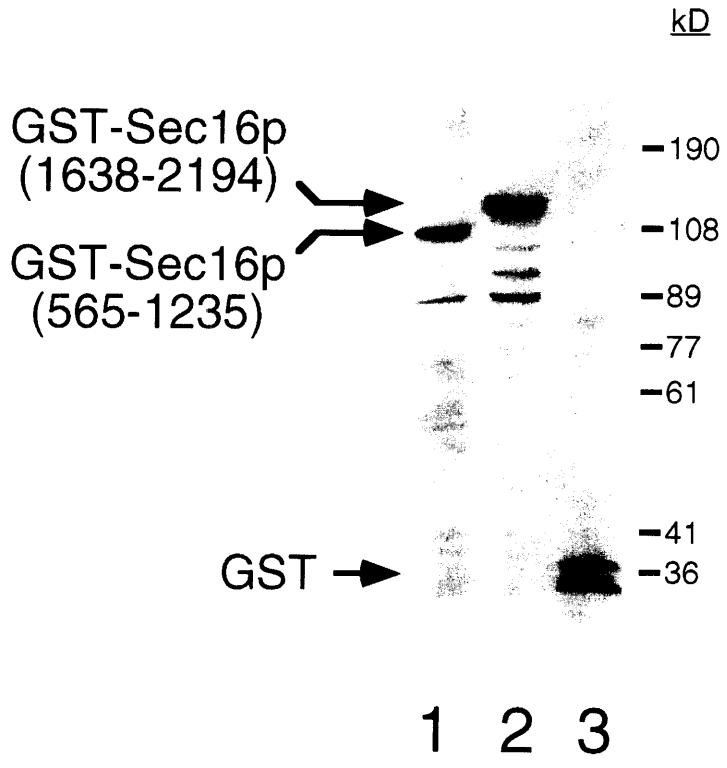

kD
GST-Sec16p
(1638-2194)
GST-Sec16p
(565-1235)
GST
190
108
89
77
61
41
36
1 2 3

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 C-terminal domain of $Sec16p_{(1638-2194)}$.

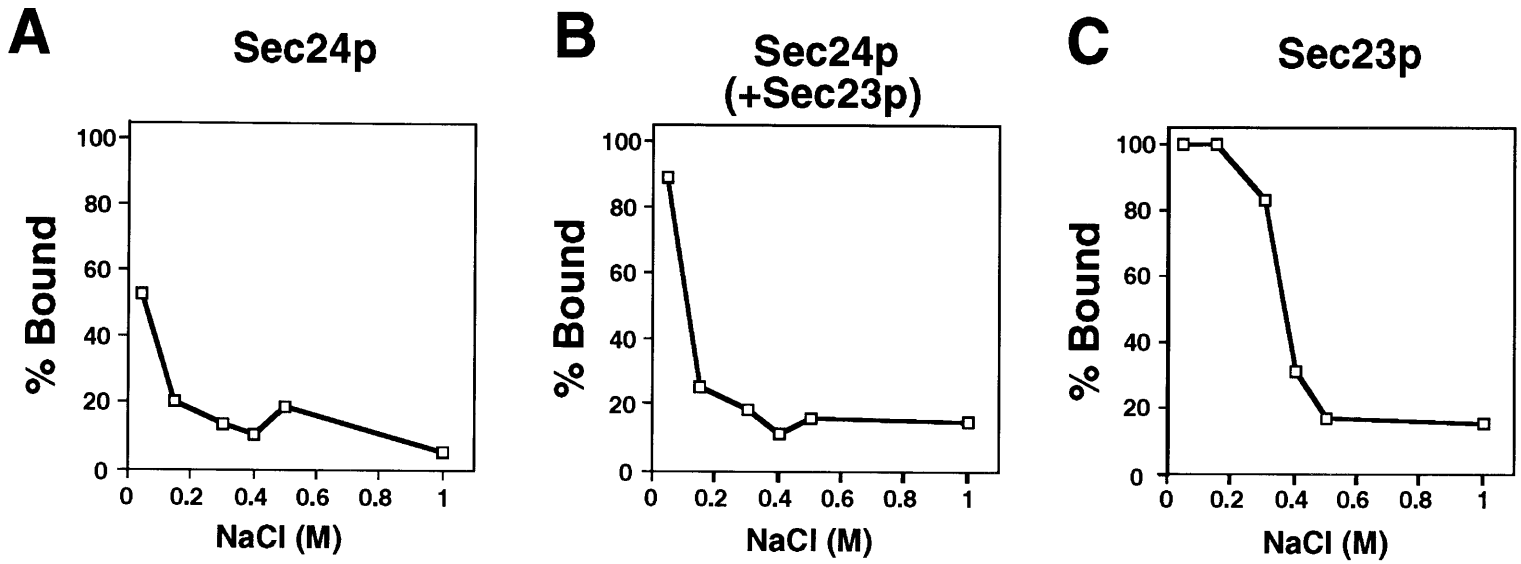

A
Sec24p
% Bound
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
NaCl (M)
B
Sec24p
(+Sec23p)
% Bound
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
NaCl (M)
C
Sec23p
% Bound
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
NaCl (M)

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

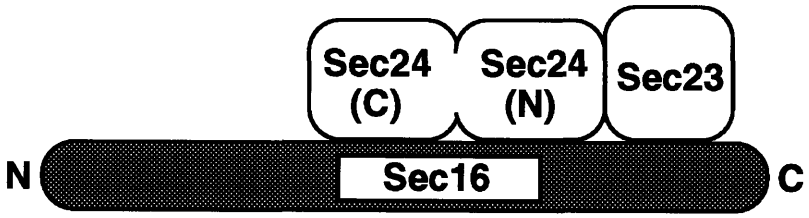

Sec24
(C)
Sec24
(N)
Sec23
N
Sec16
C

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 3

SED4 Encodes a Yeast ER Protein that Binds Sec16p and Participates in Vesicle Formation.

PREFACE

This chapter represents primarily my own work. Peter Espenshade contributed plasmids for Table II and Figure 7, and helped in the analysis of vesicles produced in vitro by gel filtration chromatography.

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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 homolog 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.

INTRODUCTION

The transport of proteins between successive organelles of the secretory pathway is mediated by vesicle carriers that bud from the membrane of the donor compartment and then fuse with the membrane of the acceptor compartment (Palade, 1975). A general feature of vesicle formation is the recruitment of proteins from the cytoplasm to the membrane for assembly of a coat on the budding vesicle (Pearse and Robinson, 1990; Rothman and Orci, 1992). In *Saccharomyces cerevisiae*, seven proteins have been identified by genetic and biochemical methods that are required for vesicle budding from the ER (Novick et al., 1980; Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990; Hicke et al., 1992; Salama et al., 1993). Five of these proteins (Sec13p, Sec31p, Sec23p, Sec24p and Sar1p) when added in soluble form to ER membranes will drive vesicle budding (Salama et al., 1993; Barlowe et al., 1994). Under the appropriate conditions, the vesicles that form have a coat that contains all five proteins (Barlowe et al., 1994). We recently found that a sixth protein, Sec16p, is also a vesicle coat protein (Espenshade et al., 1995). However, Sec16p is unlikely to be recruited to the vesicle from the cytoplasm since there is no soluble cytoplasmic pool of Sec16p. Instead, Sec16p adheres tightly to the ER membrane and may form a peripheral membrane scaffold onto which cytosolic coat proteins assemble (Espenshade et al., 1995).

An important mechanistic problem is how coat assembly on the ER membrane is regulated so that vesicle formation occurs at the proper time and place. Sec12p is a potential early regulator of vesicle assembly because Sec12p resides in the ER membrane and is required for vesicle formation, but is not incorporated into the finished vesicle structure (Nakano et al., 1988; Rexach and Schekman, 1991; Barlowe et al., 1994). The cytosolic, N-terminal domain of Sec12p catalyzes exchange of GTP for GDP on the 21 kD GTPase Sar1p

(Barlowe and Schekman, 1993). Overexpression of Sec12p increases the amount of Sar1p that can be bound to ER membranes *in vitro*, suggesting that Sec12p can recruit Sar1p to the ER membrane (d'Enfert et al., 1991b). Sar1p-GTP at the ER membrane is thought to then initiate assembly of coat proteins on the forming vesicle (Barlowe et al., 1994; Oka and Nakano, 1994).

S. cerevisiae has a second gene, *SED4*, that is closely related to *SEC12*. The N-terminal, cytosolic domain of Sec12p shares 45% amino acid identity with Sed4p but the luminal domains of these proteins appear unrelated (Hardwick et al., 1992). This similarity in sequence implies that *SED4* is involved in vesicular transport although the step in the secretory pathway where *SED4* functions has been difficult to establish. Deletion of *SED4* does not cause a pronounced growth or secretion defect and although *SED4* was isolated as a multicopy suppressor of a deletion of *ERD2*, the gene encoding the HDEL receptor in yeast, the mechanism of this suppression is not understood (Hardwick et al., 1992).

We became interested in the action of *SED4* in vesicular transport when we isolated *SED4* as a multicopy suppressor of *sec16* mutations. In this report, we show that *SED4* is involved in ER to Golgi transport and probably functions in conjunction with Sec16p and Sar1p in an early step in vesicle formation. Furthermore, we found that, although Sed4p and Sec12p are similar in structure and location, these proteins appear to engage in functionally distinct processes.

MATERIALS AND METHODS

Strains, Media and Microbiological Techniques. *Saccharomyces cerevisiae* strains are listed in Table I. Yeast media [rich medium (YPD), minimal medium (SD) and synthetic complete medium (SC)] were prepared and yeast genetic and molecular biological techniques were performed using standard methods (Kaiser et al., 1994). Yeast transformations were carried out using the lithium acetate method (Gietz and Schiestl, 1991). Transformants were selected on SC medium lacking the appropriate auxotrophic supplement. All experiments on plasmid-bearing strains were performed on at least two independent transformants. To assay loss of *URA3* marked plasmids, 10^5 cells were plated on SC medium containing 0.1% 5-fluoroorotic acid (Boeke et al., 1984).

Molecular Biological Techniques. DNA manipulations, subcloning and Southern blotting were carried out using standard methods (Sambrook et al., 1989). DNA hybridizations were performed using the ECL Nucleic Acids detection system (Amersham, Arlington Heights, IL). DNA sequencing was performed using the Sequenase kit (USB, Cleveland, OH). Site-directed mutagenesis was performed using the protocol of Kunkel (Kunkel et al., 1987). PCR was carried out using Taq polymerase (Perkin Elmer Cetus, Norwalk, CT).

Isolation and Analysis of Multicopy Suppressors of *sec16*. The YEp24 library (Carlson and Botstein, 1982) contains genomic inserts in a vector carrying the *S. cerevisiae URA3* gene and the 2μ origin of replication. Two temperature-sensitive *sec16* strains, CKY50 and CKY52, were transformed with YEp24 library DNA. A total of 35,000 (CKY50) and 13,000 (CKY52) transformants at a density of 1.5×10^3 to 1×10^4 colonies per plate were replica-plated and incubated at restrictive temperatures of 30°, 33° and 36°C (CKY50) or 33° and 36°C (CKY52).

Plasmids isolated from temperature-resistant colonies were tested for the ability to confer the temperature-resistant phenotype. A group of overlapping plasmids conferred growth up to 33°C (CKY50) and 36°C (CKY52) and represented the strongest suppressor locus. The corresponding gene was mapped to the right arm of chromosome III near the *SED4* open reading frame by hybridizing an internal restriction fragment to a Southern blot of *S. cerevisiae* chromosomes (Clontech, Palo Alto, CA) and to a set of ordered yeast genomic clones (Riles et al., 1993). The suppressing gene was shown to be *SED4* by subcloning fragments into pRS306-2μ and testing for their ability to confer temperature-resistance to CKY50.

Plasmid Constructions. Plasmids are summarized in Table II. p5007 is a YEp24 library plasmid containing *SED4*. pRH26 is the 7.4 kb *Apa*I-*Sal*I fragment of p5007 in pRS306-2μ. pRH46 is the 5.2 kb *Sac*I-*Sal*I fragment of pRH26 in pRS306-2μ. pRH107, pRH67 and pPE87 are the 5.2 kb *Sac*I-*Sal*I fragment of pRH46 in pRS316, pRS306 and pRS305-2μ, respectively. pRH121 and pRH120 are the 5.2 kb *Sac*I-*Sal*I fragment of pRH117 (see below) in pRS316 and pRS306-2μ, respectively.

Plasmids carrying truncations of *SED4* were obtained as follows: pRH26 was cut with *Spe*I and the 11.6 kb fragment was self-ligated to make pRH47. pRH46 was cut with *Apa*LI, the 5' overhang was filled in, a 3.5 kb fragment was purified and cut with *Sac*I-*Apa*I to produce a 1.8 kb fragment that was ligated into *Sac*I-*Sma*I cut pRS316-2μ to make pRH54. pRH77 contains the 1.2 kb *Xho*I-*Ssp*I fragment of pRH46 ligated into *Xho*I-*Sma*I cut pRS306-2μ. pRH78 contains the 1.2 kb *Xho*I-*Spe*I fragment of pRH77 ligated into *Xho*I-*Spe*I cut pRH26. pRH46 was cut with *Hin*dIII and the 11 kb fragment was self-ligated to make pRH128. pRH62 was constructed using two PCR steps (Horton et al., 1989; Yon

and Fried, 1989). PCR primers used were 5'-TTG TAA ATA AAG CCG TGC ACA TTG TGC TTA TAG GAG AAC TGT AA-3' (nt 1124-1101, *SED4*, and nt 1125-1104, SEC12, underlined), 5'-GGG ATT ACT TCT ATG GAT G-3' (nt 802-820, *SEC12*) and 5'-GAT GAA GAT GAA GAC GGC-3' (nt 1932-1949, *SED4*), templates used were pSEC1230 (Nakano et al., 1988) and pRH46. The PCR product was cut with *Sal*I-*Eco*RI and inserted into *Sal*I-*Eco*RI cut pRH50 (see below) to make pRH56. pRH62 is the 2.5 kb *Eco*RI fragment of pRH46 ligated into *Eco*RI cut pRH56. pRH141 is the 2.6 kb *Eco*RI fragment of pRH117 (see below) ligated into *Eco*RI cut pRH62. pRH148 is the 5.2 kb *Xho*I-*Sac*I fragment of pRH141 in pRS316.

pRH50 and pRH213 are the 3.5 kb *Xho*I-*Hin*dIII fragment of pSEC1230 in pRS306-2μ and pRS316, respectively.

Epitope-tagging *SED4* and *SEC12*. *SED4* was tagged with the haemagglutinin (HA) epitope (Kolodziej and Young, 1991) as follows: A single copy of the HA epitope was inserted before the C-terminal HDEL sequence of *SED4* using site-directed mutagenesis, resulting in *SED4-HA1*. The mutagenic oligomer consisted of 27 nt encoding the HA epitope (underlined) flanked by 20 and 27 nt complementary to the *SED4* sequence on the 5' and 3' end respectively (5'-CCG TAA ACT ACG CTG GCC TTT ACC CAT ACG ACG TCC CAG ACT ACG CTC ATG ACG AAT TGT GAA TAA CGA AAT AA-3'). Tandem repeats of the HA epitope were inserted by introducing a *Not*I site between the last nucleotide of the HA tag and the C-terminal HDEL sequence by site-directed mutagenesis. The mutagenic oligomer consisted of a *Not*I site (underlined) flanked by sequences complementary to *SED4-HA1* (5'-GAC GTC CCA GAC TAC GCT AGC GGC CGC CAT GAC GAA TTG TGA ATA ACG-3'). A cassette containing 3 HA epitopes (Tyers et al., 1993) was then inserted into the newly created *Not*I site,

creating pRH117. By DNA sequencing, pRH117 encodes *SED4* containing 7 tandem repeats of the HA epitope (*SED4-HA*).

The N-terminal domain of *SED4* was placed under control of the *GAL10* promoter and tagged at its 3' end with a c-myc epitope (myc) (Munro and Pelham, 1987) as follows: pCD43 is pRS316 with a 0.6 kb *Eco*RI-*Bam*HI fragment containing the *GAL1/GAL10* promoter region inserted into the polylinker. A 1 kb fragment encoding aa 1-346 of *SED4* plus a *Not*I site was amplified by PCR using pRH46 as a template and the following primers: 5'-AGT GAA TTC ATA ATG AGT GGC AAC TCT GC-3' (nt -3 to +17, *SED4*) and 5'-ATG GGT ACC GTC GAC CTA GCG GCC GCT TTT CCA AAT ATT TCG TAA AAT TGA TG-3' (nt 1214-1239, *SED4*). The amplified fragment was cut with *Eco*RI-*Kpn*I and ligated into pCD43, producing pRH183. A cassette encoding three copies of the myc epitope flanked by *Not*I sites was constructed using overlapping oligonucleotides (kindly provided by B. Futcher, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). The oligonucleotides were annealed, filled in and ligated into *Not*I-cut Bluescript vector (pKS$^+$) to make pRH177/23. The nucleotide sequence of the 3 x myc cassette in pRH177/23 is 5'-GCG GCC GCT CTG AGC AAA AGC TCA TTT CTG AAG AGG ACT TGA ATG GAG AAC AGA AAT TGA TCA GTG AGG AAG ACC TCA ACG GTG AGC AGA AGT TAA TAT CCG AGG AGG ATC TTA ATA GTG CGG CCG C-3'. The 3 x myc cassette of pRH177/23 was ligated into *Not*I cut pRH183, the resulting product was cut with *Pvu*I and inserted into *Pvu*I cut pRS313, creating pRH260. By DNA sequencing, pRH260 encodes a protein with 3 tandem repeats of the myc epitope plus 14 additional amino acids at the C-terminus. The N-terminal domain of *SEC12* was similarly placed under control of the *GAL10* promoter and tagged with the myc epitope. A 1 kb fragment encoding aa 1-354 of *SEC12* plus a *Not*I site was amplified by PCR using pSEC1230 as a template and the following

primers: 5'-AGT GAA TTC ACT ATG AAG TTC GTG ACA G-3' (nt -3 to +16, *SEC12*) and 5'-TGC GCT CGA GCT AGC GGC CGC TTT TAG AGA TTT TTT GTT TCA TTG AGG-3' (nt 1037-1062, *SEC12*). The amplified fragment was cut with *Eco*RI-*Kpn*I and ligated into pCD43, producing pRH186. The 3 x myc cassette was ligated into *Not*I cut pRH186, the resulting product was cut with *Pvu*I and inserted into *Pvu*I cut pRS313, creating pRH261. By DNA sequencing, pRH261 encodes a protein with 3 tandem repeats of the myc epitope plus 14 additional amino acids at the C-terminus.

Construction of a *SED4* Deletion Allele. A deletion of the entire *SED4* open reading frame (*sed4-Δ1*) was constructed by site-directed mutagenesis. The mutagenic oligomer (5'-CTT TTA AAC TTA GAA AAA CTA GCA TAA TAA TGG ATC CAA GCT TGA ATA ACG AAA TAA TAT ATA TTA ATG TTA AAT ATG-3') consisted of 32 nucleotides complementary to the 3' untranslated region of *SED4*, 10 nucleotides creating a *Hin*dIII and a *Bam*HI site (underlined), and 36 nucleotides complementary to the 5' untranslated region of *SED4*. Mutagenesis of pRH67 deleted the entire *SED4* reading frame as shown by restriction mapping. A 5 kb marker cassette (*hisG-URA3-Kan^r-hisG*) (modification of Alani et al., 1987; kindly provided by S. Elledge, Baylor College of Medicine, Houston, TX) was inserted into the newly created *Bam*HI site to make pRH73 (*sed4-Δ1::URA3*).

A diploid (CKY8 x CKY10) was transformed with a 6.9 kb, purified *Sac*I-*Sal*I fragment of pRH73. Tetrad analysis of Ura$^+$ transformants gave 2:2 segregation of *URA3*. By Southern blot analysis, the genomic *SED4* locus was deleted in four Ura$^+$ spores examined. Loss of the *URA3* marker by recombination of the hisG repeats was selected on SC medium containing 0.1% 5-fluoroorotic acid to produce *sed4-Δ1*.

Protein Extracts, Western Blotting and Cell Fractionation. Yeast protein extracts were prepared from 2-6 x 10^7 exponentially growing cells as described (Rothblatt and Schekman, 1989). Cells were lysed in 30 µl ESB (60 mM Tris HCl (pH 6.8), 100 mM DTT, 2% SDS, 10% glycerol, 0.001% Bromphenol Blue) by vigorous agitation with 0.5 mm glass beads (Sigma, St. Louis, MO). Extracts were diluted with 70 µl of ESB and 10-20 µl were resolved by SDS-PAGE (Laemmli, 1970). Western blotting was performed using standard methods (Harlow and Lane, 1988). The following antibodies were used: anti-HA antibody (12CA5 ascitic fluid; BABCO, Richmond, CA) at 1/1,000 dilution, anti-myc antibody (9E10 ascitic fluid; K. Morrison, Harvard University, Boston, MA) at 1/1,000 dilution, rabbit anti-CPY antibody (gift of R. Schekman) at 1/5,000 dilution, HRP-coupled sheep anti-rabbit Ig (Amersham) at 1/10,000 dilution and HRP-coupled sheep anti-mouse Ig (both Amersham) at 1/10,000 dilution. Blots were developed using the ECL system (Amersham). Cell fractionation was performed as described (Espenshade et al., 1995) using CKY295.

Radiolabeling and Immunoprecipitations. Cells were grown in selective SC medium supplemented with 2% glucose and then shifted to the indicated temperatures 2 h before labeling. Tunicamycin treatment and temperature-shift experiments of the *dpm1-6* mutant were performed as described (Orlean et al., 1991). 2-6 x 10^7 exponentially growing cells (1-3 OD_{600} units) were radiolabeled in supplemented SD medium by incubating with 30 µCi [^{35}S]-methionine per OD_{600} unit (Express protein labeling mix (NEN, Boston, MA), spec. activity 1200 Ci /mmol). Samples were chased by the addition of 1/100 volume of a solution containing 0.1 M ammonium sulfate, 0.3% cysteine, 0.4% methionine. Labeled samples of 1 OD_{600} units of cells were collected into

chilled tubes containing an equal volume of 40 mM sodium azide. Protein extracts were prepared in 30 µl ESB by vigorous agitation with glass beads. Extracts were diluted with 1 ml IP buffer (50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.2% SDS), absorbed for 20 min with 50 µl 10% *S. aureus* cells (Sigma) and cleared by centrifugation at 12,000 x *g* for 5 min. 0.5 µl anti-CPY or anti-HA antibody was added and extracts were rotated for 1 h at 25°C. Immune complexes were collected by adding 30 µl 50% Protein A Sepharose (Pharmacia, Piscataway, NJ) per sample and incubating for 1 h at 25°C. Protein A Sepharose beads were washed twice with IP buffer and once with detergent-free IP buffer (50 mM Tris HCl (pH 7.4), 150 mM NaCl). Protein was released into 30 µl ESB by heating to 100°C for 2 min. 10 µl of the supernatant was separated by SDS-PAGE and visualized by fluorography (Harlow and Lane, 1988).

Two-Hybrid Protein-Protein Interaction Assay. Sed4p and Sec12p were tested for binding to Sec16p *in vivo* as described by Gyuris et al., 1993. The N-terminal domain of Sed4p or Sec12p was fused to the acidic activation domain in pJG4-5 as follows. A fragment encoding aa 1-347 of *SED4* was amplified by PCR using pRH46 as a template and the following primers: 5'-AGT GAA TTC ATA ATG AGT GGC AAC TCT GC-3' (nt -3 to +17, *SED4*) and 5'-CTA GTC GAC CTA TTT CCA AAT ATT TCG TAA AAT TGA TG-3' (nt 1038-1016, *SED4*). The corresponding fragment encoding aa 1-354 of *SEC12* was amplified similarly using pSEC1230 as a template and the following primers: 5'-AGT GAA TTC ATG AAG TTC GTG ACA GCT AG-3' (nt 1-20, *SEC12*) and 5'-TGC GCT CGA GCT ATT TAG AGA TTT TTT GTT TCA TTG AGG-3' (nt 1062-1037, *SEC12*). Fragments were cut with *Eco*RI-*Sal*I (*SED4*) or *Eco*RI-*Xho*I (*SEC12*) and ligated into *Eco*RI-*Xho*I cut pJG4-5 to make pRH154 and pRH155, respectively. pPE58, 59 and 74 are plasmids encoding aa 1645-2194, aa 1-824 and aa 447-1737 of

SEC16 fused to the *lexA* DNA binding domain in pEG202 (Espenshade et al., 1995).

Two-hybrid interactions were tested in EGY40 (Golemis and Brent, 1992) transformed with the appropriate plasmids. *LacZ* expression was tested by patching 4 to 8 transformants on SC medium (pH 7.0) lacking the appropriate amino acids and supplemented with 2% galactose and 40 mg/l X-gal. For β-galactosidase assay, cells were grown to exponential phase in selective medium containing 2% raffinose, then galactose was added to 2% and growth was continued for 10 h. Extracts were prepared and assayed as described (Kaiser et al., 1994). Protein concentrations in the extracts were determined using the Bradford Assay (Biorad, Melville, NY). 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].

Binding to GST-fusions. The C-terminus of Sec16p (aa 1688-2194) was fused to the glutathione-S-transferase gene (GST) expressed from the *GAL1* promoter (pRD56, a kind gift of Dr. Ray Deshaies) to create pPE122. The N-terminal domains of Sed4p and Sec12p were expressed from the *GAL10* promoter and tagged with the myc epitope as described above (pRH260 and pRH261). pPE122 and either pRH260 or pRH261 were transformed into CKY289. For controls, CKY289 carrying pRD56 and either pRH260 or pRH261 were used.

Cells were grown to exponential phase in selective medium containing 2% raffinose, galactose was added to 2%, and extracts were prepared 4 h later. 4 x 10^7 cells were suspended in 40 µl CoIP buffer (20 mM HEPES (pH 6.8), 80 mM potassium acetate, 5 mM magnesium acetate, 0.1% Triton X-100) containing 2.5 x 10^{-4} U/ml α2-macroglobulin, 1 mM PMSF, 0.5 ng/ml leupeptin, 10 ng/ml E64 and 0.4 ng/ml aprotinin (all Boehringer, Indianapolis, IN) and were lysed by

vigorous agitation with 0.5 mm glass beads 4 x 20 sec with 1 min intervals on ice. Extracts were diluted to 1 ml with CoIP buffer with protease inhibitors, and the lysate was cleared by centrifugation at 13,000 x *g* for 5 min. Glutathione Sepharose 4B beads (Pharmacia, Piscataway, NJ) were added and samples were incubated for 1 h at 25°C. The beads were washed 3 times with CoIP buffer and once with detergent-free CoIP buffer. Proteins were released from the beads by boiling in 30 µl ESB. Total protein extracts were prepared from 2 x 10^7 cells lysed in ESB by agitation with glass beads.

Immunofluorescence. Indirect immunofluorescence was performed essentially as described by Pringle et al., 1991. Cells were fixed by adding formaldehyde (final concentration of 3.7%) to the medium and incubating for 2 h at 25°C. Fixed cells suspended in 0.1 M potassium phosphate (pH 7.2) were spheroplasted with 50 U lyticase for 30 min at 37°C. Incubations in primary or secondary antibody were for 1 h and were performed in a humid chamber at 25°C. The antibodies and concentrations used were: 12CA5 at a 1/5,000 dilution, anti-BiP polyclonal antiserum (kind gift of M. Rose, Princeton University, NJ) at 1/1,000 dilution, FITC-coupled goat anti-rabbit IgG and rhodamine-coupled goat anti-mouse IgG (both Boehringer) at 1/300 dilution. Cells were mounted in medium containing DAPI and *p*-phenylenediamine. Images were recorded on a Zeiss axioscope using Kodak T-Max 400 film developed according to the manufacturer's specifications.

Electron Microscopy. Electron microscopy was performed as described in Kaiser and Schekman, 1990. Wild type (CKY291), *sed4-Δ1 sar1-5* (CKY293) and *sec17-1* (CKY54) cells were grown to exponential phase in YPD at 24°C and shifted to 38°C for 2 h prior to fixation. Cells were fixed for electron microscopy

with potassium permanganate. To count vesicles, random well-stained sections were selected, photographed at a magnification of 13,000x, and vesicles seen on the negative were counted. Vesicle counts were normalized to cell volume by measuring the area of the cell section and assuming a section thickness of 90 nm. 29 and 33 cell sections were counted for the *sed4-Δ1 sar1-5* and the *sec17-1* strain, respectively. Data are expressed as mean ± standard error of the mean.

***In Vitro* Vesicle Synthesis.** Membranes and cytosol used in the vesicle synthesis reaction were prepared as previously described (Wuestehube and Schekman, 1992) using CKY295 as a source of membranes and CKY93 as a source of cytosol. Standard vesicle synthesis reactions of 500 µl contained 100 µg of membranes, 1.2 mg of cytosol prepared in the absence of added guanine nucleotide, 1mM 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, 5mM MgOAc, 250 mM sorbitol) with protease inhibitors (1 mM PMSF, 0.5 ng/µl leupeptin, 1 µM pepstatin). 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. Donor membranes were removed by centrifugation at 32,000 rpm for 10 min at 4°C in a Beckman TLA100.3 rotor. Vesicles were pelleted from this medium speed supernatant by centrifugation at 60,000 rpm for 30 min at 4°C in a Beckman TLA100.3 rotor. Vesicle pellets were solubilized in 30 µl ESB and proteins were analyzed by Western blotting.

Vesicles formed *in vitro* were fractionated by gel filtration on a 14 ml (1 x 18 cm) Sephacryl S-1000 column equilibrated in reaction buffer as described (Barlowe et al., 1994). A 0.7 ml sample of medium speed supernatant from two 0.5 ml reactions was applied to the column, eluted in 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 Beckman TLA100.3 rotor. Proteins solubilized in 30 μl ESB were analyzed by Western blotting.

Cloning and Sequence Determination of *sar1-5*. *sar1-5* was cloned by gap repair as follows: pRH259 contains the *SAR1* gene on a 1.3 kb *Hin*dIII-*Sal*I fragment of pSEC1210 (Nakano and Muramatsu, 1989) inserted into pRS316 with a deletion from the *Eco*RI to the *Not*I sites of the polylinker. A *sar1-5 sed4-Δ1* double mutant was transformed with plasmid pRH259 cut with *Eco*RI and *Bam*HI to produce a gap covering the *SAR1* coding sequence. Gap-repaired plasmids carrying the mutation (pRH262) were identified by their inability to suppress the temperature-sensitivity of *sec16-2* and *sec23-1* strains. The mutational change in a plasmid carrying *sar1-5* was identified by sequencing with synthetic oligonucleotide primers. *SAR1* and *sar1-5* were placed on a 2μ plasmid by inserting the 1 kb *EcoR*I-*Hind*III fragment of pRH259 and pRH262 into pRS306-2μ, creating pRH280 and pRH279.

RESULTS

***SED4* is a Multicopy Suppressor of *sec16* Mutations.** *sec16-2* mutants fail to grow at temperatures above 30°C (Fig. 1, column 1). To identify genes that interact with *SEC16*, we screened a *S. cerevisiae* genomic DNA library in a multicopy (2μ) vector for plasmids that permit temperature-sensitive (Ts) *sec16* mutants to grow at restrictive temperatures. One set of overlapping plasmids was recovered that suppressed the growth defect of *sec16-2* cells up to 36°C (Fig. 1, column 2). Subcloning and sequencing identified the suppressing locus as *SED4*.

SED4 overexpression partially suppressed the Ts growth defect of all four *sec16* alleles (not shown). However, *SED4* overexpression could not bypass a *sec16* null allele (*sed16-Δ1::TRP1*) as demonstrated in the following plasmid shuffling experiment. CKY234 carries a chromosomal *sed16-Δ1::TRP1* allele and a *URA3* marked plasmid containing *SEC16*. CKY234 transformed with *SED4* on a 2μ, *LEU2* marked plasmid (pPE87) could not grow without the *URA3* marked *SEC16* plasmid, whereas CKY234 transformed with *SEC16* on a *LEU2* marked plasmid (pPE8) could grow without the *URA3* marked plasmid.

We examined the ability of *SED4* overexpression to suppress Ts mutations in other secretion genes. The *sec* and *bet* mutants listed in Table I were transformed with either *SED4* on a multicopy plasmid (pRH46) or the 2μ vector alone, and tested for growth at 30°, 33°, 36° and 38°C. Overexpression of *SED4* partially suppressed the growth defect of *sec2-41* at 33°C, but had no effect on the growth defect of any of the other mutants tested. Importantly, overexpression of *SED4* did not suppress the growth defect of a strain carrying a Ts mutation in *SEC12*, the gene most like *SED4*.

The ability of *SED4* overexpression to suppress the secretion defect of *sec16-2* was examined by following the maturation of the vacuolar enzyme

carboxypeptidase Y (CPY). Covalent modifications of CPY in the ER, the Golgi, and the vacuole mark the early events in the secretory pathway (Stevens et al., 1982). Strains were grown at 32°C for 2 h, labeled for 5 min, and then chased. In a *sec16-2* strain, none of the ER-form (p1) of CPY was converted into the mature, vacuolar form (m) even after 30 min (Fig. 2, lanes 1-5), whereas the same strain containing *SED4* on a *2μ* plasmid allowed maturation of CPY (Fig. 2, lanes 6-10). Thus, the suppression of the secretion defect of *sec16-2* by *SED4* parallels the suppression of the growth defect.

The Conserved N-terminal and Transmembrane Domains of Sed4p Confer Suppression of the Growth Defect of *sec16-2* Strains. To identify the portion of *SED4* required for suppression of *sec16*, truncations of *SED4* were tested. By comparison to *SEC12*, *SED4* is comprised of an N-terminal cytosolic domain, a transmembrane domain, and a C-terminal luminal domain. *SED4* alleles with either a partial (*sed4-T1*) or a complete (*sed4-T2*) deletion of the C-terminal domain suppressed *sec16-2* almost as well as wild type *SED4* demonstrating that this domain is not necessary for suppression (Fig. 3). The N-terminal and transmembrane domains of Sed4p were required for suppression, since deletion of a 39 amino acid segment of the N-terminal domain (*sed4-T4*) or deletion of the transmembrane domain (*sed4-T3*) completely abolished the ability to suppress *sec16-2* (Fig. 3). To demonstrate that *sed4-T4* is expressed at levels similar to *SED4*, we constructed an epitope-tagged allele, *sed4-T4-HA*, that contains the HA epitope at the same position as *SED4-HA* (see below). By Western blotting, the levels of Sed4-T4-HAp were identical to Sed4-HAp (not shown).

The function of the C-terminal luminal domain of Sed4p was further examined by fusing it to the N-terminal and transmembrane domains of Sec12p and testing this chimera for suppression of either *sec16-2* or *sec12-4*. The

chimera behaved like wild type *SEC12*: *SEC12-SED4* on either a low or a high copy plasmid did not suppress *sec16-2*, but complemented both *sec12-4* and a chromosomal deletion of *sec12* (Fig. 3 and data not shown). These results demonstrate that the function of *SED4* is specified by its conserved N-terminal and transmembrane domains.

Sed4p is an O-glycosylated ER Membrane Protein. Sed4p was epitope-tagged by inserting seven copies of the haemagglutinin (HA) epitope before the C-terminal HDEL sequence (see Materials and Methods). Epitope-tagged *SED4* appeared to be functional since *SED4-HA* suppressed *sec16-2* to the same extent as untagged *SED4* (not shown). Immunoblots probed with anti-HA antibodies detected a protein that migrated more slowly than a 190 kD molecular weight standard (Fig. 4A, lane 1). As expected for Sed4p-HA, this band was more abundant in a strain overexpressing Sed4p-HA (Fig. 4A, lane 2) and was not present in a strain expressing untagged *SED4* (Fig. 4A, lane 5).

An epitope-tagged Sec12-Sed4p fusion protein (Fig. 3) was useful for estimating the abundance of Sec12p relative to Sed4p. Since both Sed4p-HA and the chimera were tagged at the same position and migrated similarly on SDS-PAGE, they were likely to be detected with equal efficiency by Western blotting. Sed4p-HA expressed from either a high or a low copy vector was 5 to 10 times more abundant than Sec12-Sed4p-HA, expressed from the same vector (Fig. 4A, compare lanes 1 and 3 and lanes 2 and 4).

The difference between the observed molecular weight of Sed4p-HA (>190 kD) and the molecular weight predicted from the amino acid sequence (117kD) prompted us to examine possible modifications of Sed4p. The C-terminal, luminal domain of Sed4p contains three potential N-linked glycosylation sites and is rich in serine and threonine residues that could accept O-linked

glycosylation. Unglycosylated Sed4p-HA was produced in PRY303, a strain that carries a Ts mutation in dolichol phospho mannose synthase, an enzyme required for both N- and O-linked glycosylation (Orlean, 1990). Sed4p-HA, immunoprecipitated from PRY303 cells labeled at the restrictive temperature, migrated more rapidly than Sed4p-HA expressed in wild type cells (Fig. 4B, lane 4), indicating that Sed4p-HA is a glycoprotein. Treatment of cells with tunicamycin, an inhibitor of N-linked glycosylation, or treatment of extracts with EndoH to remove N-linked carbohydrate chains had no effect on the electrophoretic mobility of Sed4p-HA (Fig. 4B, lane 5 and data not shown), indicating that Sed4p-HA is modified primarily by O-glycosylation. The discrepancy between the migration of Sed4p-HA without carbohydrate modifications (190 kD) with that predicted from the amino acid sequence (117 kD) is probably due to anomalous migration on SDS-PAGE since Sed4p-HA expressed in bacterial cells also migrated at 190 kD (not shown).

Sed4p-HA behaved as an ER-membrane protein on cell fractionation. A large fraction of Sed4p-HA in a cell lysate pelleted at 500 x *g* and the remainder pelleted at 10,000 x *g* (not shown). As expected for an integral membrane protein, Sed4p-HA was partially solubilized from the 10,000 x *g* pellet by treatment with 1% Triton-X 100, but was not released by treatment with 2.5 M urea, 0.5 M NaCl, or sodium carbonate (pH 11) (not shown).

The intracellular location of Sed4p-HA was further examined by indirect immunofluorescence. Figure 5 (top panel) shows diploid cells expressing *SED4-HA* from a high copy plasmid stained with anti-HA antibody. The staining was chiefly at the nuclear periphery with extensions into the cytoplasm and around the periphery of the cell body. This pattern is typical for proteins located in the ER (Rose et al., 1989). The anti-HA staining pattern was identical to the anti-BiP staining observed in a double labeling experiment (Fig. 5, bottom panel),

indicating that Sed4p-HA is distributed throughout the ER. A similar, though weaker, staining was seen for Sed4p-HA expressed from a low copy plasmid, while no staining was apparent in a control strain transformed with untagged *SED4* (not shown).

Sed4p is Excluded from ER Vesicles Produced *In Vitro*. To investigate whether Sed4p is present on vesicles that have budded from the ER, we followed the fate of Sed4p-HA in a cell-free ER budding reaction (Wuestehube and Schekman, 1992). ER membranes were isolated from a *sed4* deletion strain expressing Sed4p-HA from a low copy plasmid. Vesicles were produced by incubating these membranes with a guanine nucleotide and cytosol. Vesicles that had formed *in vitro* were isolated by first removing the donor membranes by centrifugation at medium speed and then pelleting the vesicles at high speed. Sec22p was used as a vesicle marker protein and about 10% of Sec22p was recovered in the high speed pellet (HSP) after incubation of donor membranes at 20°C with GTP and cytosol (Fig. 6). Incubation at 4°C, in the presence of apyrase, or in the absence of cytosol decreased the amount of Sec22p in the HSP by 10-fold, while incubation in the presence of GMP-PNP, a non-hydrolyzable GTP-analog, reduced vesicle formation by about two-fold. The conditions that promote vesicle formation in these reactions, and the efficiency of vesicle formation are consistent with those found previously (Rexach et al, 1994). Sed4p-HA was detectable in both ER membrane and vesicle fractions, but only 0.1-0.5% of membrane-bound Sed4p-HA was released into the vesicle fraction at 20°C as compared to 10% of the input Sec22p. Even less (0.01%) was released on incubation at 4°C, without GTP or with GMP-PNP. To determine whether the small amount of Sed4p-HA released in a GMP-PNP reaction was present on ER to Golgi transport vesicles or was associated with another type of membrane, the

vesicle fraction from a GMP-PNP reaction was fractionated further by gel-filtration chromatography (Barlowe et al., 1994). Most Sed4p-HA eluted before Sec22p on a Sephacryl S-1000 column (data not shown). Thus, the small amount of Sed4p that is released from the ER is not in transport vesicles and Sed4p, like Sec12p, appears to be largely excluded from budded vesicles.

The N-terminal domain of Sed4p binds to the C-terminal domain of Sec16p. The genetic interaction between *SED4* and *SEC16* suggested that their products might physically associate. As an initial test of this possibility we used the two-hybrid system (Fields and Song, 1989; Gyuris et al., 1993). The N-terminal domain of *SED4* was fused to an acidic transcription activation domain and tested for interaction with each of three overlapping parts of *SEC16* fused to the *lexA* DNA binding domain. Interaction was scored by the ability of the *lexA* DNA binding domain and the acidic activation domain to be brought together to drive transcription of a *lacZ* reporter gene. A strong interaction was detected for the combination of the N-terminal domain of *SED4* and the C-terminal domain of *SEC16* (Table III). This interaction was specific for *SED4* since a parallel test of the N-terminal domain of *SEC12* gave no interaction (Table III). The possibility that *SEC12* failed to interact because of poor expression was tested by evaluating protein levels by Western blotting with antibodies against the HA-tag present in the acidic activation domain. Both Sec12p and the Sed4p fusion proteins were present at comparable levels, indicating that the results of the two-hybrid test do reflect the inability of Sec12p to interact with Sec16p.

Sed4p and Sec16p were also tested for binding in cell extracts. Since Sec16p (Espenshade et al., 1995) and Sed4p are both insoluble, we tested association of only the putative interacting regions expressed in soluble form. The N-terminal domains of Sed4p and Sec12p were tagged with the myc epitope

and expressed from the *GAL10* promoter (*SEC12N-MYC* and *SED4N-MYC*). These epitope-tagged constructs were first tested for functionality as follows. We found that overexpression of either the Sec12p N-terminal domain or the Sed4p N-terminal domain has a dominant negative effect and exacerbates the temperature-sensitivity of *sec12-4* and other mutants defective in vesicle formation (d'Enfert et al., 1991a; Gimeno and Kaiser, unpublished observations). *SEC12N-MYC* and *SED4N-MYC* both inhibited the growth of *sec12-4* to the same extent as untagged controls, indicating that addition of the epitope did not interfere with function. These tagged domains were tested for binding to the C-terminal domain of Sec16p fused to glutathione-S-transferase (GST) and expressed from the *GAL1* promoter (*GST-SEC16C*). GST-Sec16Cp and associated proteins were isolated by affinity to glutathione beads from extracts prepared from yeast cells expressing *GST-SEC16C* and either *SED4N-MYC* or *SEC12N-MYC*. Sed4Np-Myc, but not Sec12Np-Myc, associated with GST-Sec16Cp bound to glutathione beads as detected by Western blotting using the anti-myc antibody (Fig. 7, lanes 1 and 2). The binding of Sed4Np-Myc was dependent on the presence of Sec16Cp since none associated with GST alone (Fig. 7, lanes 3 and 4). Thus, the binding experiments gave the same result as the two-hybrid tests; Sed4p can bind to the C-terminal domain Sec16p and a parallel interaction is not seen for Sec12p.

Deletion of *SED4* Slows Transport of CPY from the ER to the Golgi. The genetic and physical interactions between Sed4p and Sec16p prompted us to examine more carefully the phenotypes of a chromosomal deletion of *SED4* (*sed4-Δ1*). Previously, no growth or secretion defect was found in a *SED4* disruption strain (Hardwick et al., 1992). Consistent with these data, *sed4-Δ1* cells grew as well as isogenic wild type cells at a range of different temperatures

(15°, 25°, 38° or 40°C) and showed no accumulation of the ER form of CPY by Western blotting (not shown). However, *sed4-Δ1* strains did not grow at 41°C, although wild type strains grew slowly at this temperature. This growth defect of *sed4-Δ1* strains was complemented by *SED4* and could be suppressed by *SAR1*, *SEC16* or *SEC23* on a low copy vector, but not by *SEC13* or *SEC12* (not shown). Complementation of *sed4-Δ1* at 41°C provided another test of *SED4* function and was also used to establish the importance of the N-terminal domain (Fig. 3).

We examined the kinetics of secretion of CPY in *sed4-Δ1* cells at 38°C (Fig. 8). In wild type, 50% of the ER (p1) form of CPY was converted to the Golgi (p2) form after 4 min of chase, and CPY was completely converted to the mature vacuolar (m) form after 8 to 10 min of chase (Fig. 8A, lanes 7-12). In *sed4-Δ1*, p1 CPY persisted beyond 10 min of chase indicating slowed transport from the ER (Fig. 8A, lanes 1-6). Quantitation of the rate of conversion of p1 CPY to mature form (Fig. 8B) gave a half-life of p1 CPY of 7.1 min in a *sed4-Δ1* strain compared to 4.4 min in wild type. This transport defect in *sed4-Δ1*, although subtle, was highly reproducible, and a 1.6- to 2-fold lower transport rate from the ER to the Golgi of *sed4-Δ1* cells was found in four independent experiments.

Deletion of *SED4* Exacerbates Vesicle Formation Mutations. Synthetic lethal interactions between genes that affect the secretory pathway have been found among genes required for protein translocation across the ER membrane (Rothblatt et al., 1989), genes required for vesicle formation at the ER (Kaiser and Schekman, 1990), genes required for vesicle fusion with the Golgi (Kaiser and Schekman, 1990; Newman et al., 1987), and genes required for fusion of secretory vesicles with the plasma membrane (Salminen and Novick, 1987). Because such interactions have only been detected between genes that affect the same step of the pathway, systematic tests for synthetic lethality can often

define the step where a gene product acts. To test the interactions of *sed4-Δ1*, a *URA3*-marked *sed4-Δ1* strain was crossed to a panel of Ts secretion mutants. The temperature-sensitivity of mutations in each of four genes required for vesicle formation at the ER (*sec12-4*, *sec13-1*, *sec16-2*, *sec23-1*) was significantly increased, when combined with *sed4-Δ1::URA3* (Table IV). Importantly, these effects were specific for vesicle formation functions since *sed4-Δ1* did not increase the temperature-sensitivity of the mutants required for vesicle fusion (*sec17-1*, *sec18-1*, *sec22-3*, Table IV and not shown) or any other secretion mutations (*sec20-1*, *sec21-1*, *sec2-41*, *sec4-8*, *sec7-1*, *sec8-9*, not shown). This pattern of synthetic lethal interactions shows that only defects in vesicle formation were made more severe by the absence of Sed4p and therefore points to a role for *SED4* in vesicle formation at the ER.

Isolation of *sed4* as an Early Secretory Pathway Mutant. Perhaps the most convincing demonstration that *SED4* is important for ER to Golgi transport came from the isolation of a *sed4* mutant in a general screen for new secretion mutants. We examined a collection of 1,800 random Ts mutants for accumulation of the ER forms of CPY and invertase by Western blotting (E. Holzmacher and C. Kaiser, unpublished). After backcrossing and complementation testing, Ts mutations in about 15 new genes required for ER to Golgi transport have been identified. Segregation analysis of one of these mutants, designated EH874, revealed that its growth and secretion defect was caused by mutations in two unlinked genes. Analysis of crosses of EH874 to wild type showed that the double mutant segregants were temperature-sensitive at 38°C, one of the single mutants was temperature-sensitive at 41°C, and the other single mutant showed no growth defect. The mutation that caused temperature-sensitivity at 41°C was shown to be an allele of *SED4* because it failed to

complement the growth defect of *sed4-Δ1* at 41°C and was completely linked to *sed4-Δ1* in tetrad analysis. This allele was designated *sed4-1* and in all the phenotypic tests we performed behaved the same as *sed4-Δ1.* The other mutation in EH874 was phenotypically silent on its own, but was needed to confer temperature-sensitivity on *sed4-1.* Because *SAR1* on a low copy plasmid complemented the temperature-sensitivity of EH874, we suspected that the second mutation might be an allele of *SAR1.* Linkage to *SAR1* was tested by crossing a *sed4-Δ1* strain in which the *SAR1* locus was marked with *URA3* (CKY282) to EH874. Tetrad analysis of the resulting diploids demonstrated that the mutation that caused temperature-sensitivity was tightly linked to *SAR1.* The effect of this allele, designated *sar1-5*, on growth and secretion is shown in Figure 9. *sar1-5* alone had no growth or secretion defect whereas *sar1-5* combined with *sed4-Δ1* showed a severe growth defect and a complete block in transport of CPY to the Golgi at 38°C. The simplest explanation for these results is that Sed4p is needed for efficient utilization of Sar1p, and that in the absence of Sed4p the subtle defect caused by the *sar1-5* mutation produces a strong secretion defect.

The *sar1-5* allele was recovered from the chromosome by gap-repair of a *SAR1* plasmid. The DNA sequence of *sar1-5* revealed a change from G to T at nucleotide 533, replacing methionine 41 with isoleucine. Methionine 41 occurs in Sar1 proteins from all organisms examined so far and is located in a highly conserved region immediately following the G1 guanine nucleotide binding domain and preceding the putative effector binding domain (Kuge et al., 1994). Mutations in this region have not been previously characterized in either Sar1p or its closest homologue Arf1p.

The *sed4-Δ1 sar1-5* Double Mutant Accumulates ER Membranes but not Vesicles. The finding that deletion of *SED4* in a *sar1-5* background causes a temperature-sensitive ER to Golgi transport defect allowed us to examine in more detail the step at which Sed4p functions. Mutants that block ER to Golgi transport fall into two morphological classes: mutants defective in fusion of ER-derived vesicles with the Golgi accumulate ER membranes and a large number of 50 nm vesicles, whereas mutants defective in vesicle formation accumulate only ER membranes (Kaiser and Schekman, 1990). We examined the morphology of the *sed4-Δ1 sar1-5* mutant after growth at 38°C for 2 h to impose a complete block in ER to Golgi transport (see Fig. 9). Cells were fixed with potassium permanganate to highlight membranes and were viewed by electron microscopy. *sed4-Δ1 sar1-5* double mutant cells accumulated excess ER membranes, visible as extra layers of membrane throughout the cell (Fig. 10). To determine whether *sed4-Δ1 sar1-5* cells also accumulated 50 nm vesicles, we counted vesicles in random cell sections. The average number of vesicles per μm^3 cell volume in *sed4-Δ1 sar1-5* cells was 7.4 ± 1.1. This value is similar to that previously reported for other mutants defective in vesicle formation (Kaiser and Schekman, 1990). To establish our ability to detect vesicles in this experiment, we counted vesicles in a mutant defective in vesicle fusion (*sec17-1*) that was grown at the restrictive temperature and was fixed for microscopy in parallel. As expected, the *sec17-1* mutant accumulated vesicles (19.8 ± 2.2 vesicles per μm^3 cell volume). This result implies that the *sed4-Δ1 sar1-5* double mutation blocks vesicle formation at the ER, and is consistent with the genetic interactions between *SED4* and vesicle formation genes and with the localization of Sed4p to the ER membrane, but not to vesicles.

The *sar1-5* Mutation Disrupts Interaction of *SAR1* with *SEC16* but not *SEC12*. An important test for *SAR1* function is the ability to suppress mutations in other *SEC* genes. *SAR1* was first isolated because overexpression of *SAR1* suppresses *sec12* mutations (Nakano and Muramatsu, 1989). Overexpression of *SAR1* also suppresses *sec16* and *sec23* mutations, although the mechanistic relationship to *sec12* suppression is not known (Nakano and Muramatsu, 1989; Oka and Nakano, 1994). To explore the nature of the *sar1-5* mutation, we tested *sar1-5* expressed from either a low (CEN) or a high (2μ) copy plasmid for the ability to suppress different *sec* mutations. The *sar1-5* mutation disrupted the interaction of *SAR1* with *SEC16* and *SEC23*, since *sar1-5* on either low or high copy plasmids did not suppress *sec16-2* or *sec23-1* mutations (Table V). In contrast, *sar1-5* suppressed the temperature-sensitivity of *sec12-4* to the same degree as wild type *SAR1* (Table V). Thus, the *sar1-5* allele allowed the function of *SAR1* needed to suppress *sec12* mutations to be distinguished from the function(s) needed to suppress *sec16* and *sec23* mutations.

In tests of *sar1-5* for synthetic lethal interactions, *sar1-5* exacerbated the temperature-sensitivity of *sec16-2* , *sec13-1* and *sec23-1*(and of *sed4-Δ1* as described above), but had no effect on the growth of *sec12-4* (Table IV). Again, these results indicate that *SAR1* has at least two different functions. One function involves interaction with *SED4*, *SEC16* and *SEC23* and is disrupted by *sar1-5*, while the other function involves interaction with *SEC12* and is not affected by *sar1-5*.

DISCUSSION

The major conclusion of this study is that *SED4* encodes an important, but not essential, component of the machinery that assembles transport vesicles at the ER membrane. This conclusion rests on five findings. (1) Strains with a chromosomal deletion of *SED4* exhibit a two-fold reduction in the rate of transport of the marker protein CPY from the ER to the Golgi. (2) The cytosolic domain of Sed4p binds to the C-terminal domain of Sec16p, an ER and vesicle protein that is required for transport vesicle budding *in vivo*. (3) Sed4p is located in the ER membrane but not in vesicles, and therefore binding to Sec16p must take place on the ER membrane. (4) Increased dosage of *SED4* suppresses *sec16* mutations. (5) Deletion of *SED4* exacerbates mutations in genes known to participate in vesicle budding (*SEC16*, *SEC12*, *SEC13*, *SEC23* and *SAR1*), but not mutations that affect later steps in the secretory pathway. The interaction with *SAR1* is particularly striking since the *sar1-5* mutation alone is phenotypically silent, but when combined with *sed4-Δ1* shows a strong secretion block.

An important clue to the mechanism of *SED4* function is the binding of the cytosolic domain of Sed4p to the C-terminal domain of Sec16p. This interaction was detected both by two-hybrid assay and by binding experiments in cell extracts where the two interacting domains were expressed as soluble proteins. An internal control for the specificity of the interaction between Sed4p and Sec16p is provided by comparing binding of Sec16p to the cytosolic domains of Sed4p and Sec12p. The binding that we observe is specific to Sed4p because the cytosolic domain of Sec12p, which must have a similar structure to the cytosolic domain of Sed4p, does not interact with Sec16p by either two-hybrid or solution binding assays. Furthermore, two-hybrid tests between Sed4p and regions of Sec16p other than the C-terminal domain gave no interaction and a

deletion that removed 250 amino acids from the C-terminus of Sec16p disrupted the ability to interact with Sed4p (not shown). These results show a specific association between the cytosolic domain of Sed4p and the C-terminal domain of Sec16p. Since both proteins are located at the ER membrane this is presumably where they interact.

Genetic tests provide strong evidence that *SED4* is important for the proper function of *SEC16*. When vesicle formation is impaired by *sec16* mutation, increased dosage of *SED4* restores function, whereas deletion of *SED4* increases the severity of the defect. Since the activity of *SEC16* varies according to both increased and decreased dosage of *SED4*, and since Sed4p binds to Sec16p, we conclude that *SED4* is almost certainly needed for proper function of *SEC16* in vesicle formation. *SAR1* shows genetic interactions with *SEC16* that are similar to the ones observed between *SED4* and *SEC16.* Increased dosage of *SAR1* suppresses *sec16* mutations (Nakano and Muramatsu, 1989), and *sec16-2* is lethal at 27°C when combined with *sar1-5*. *SAR1* also interacts genetically with *SED4*. We show that increased dosage of *SAR1* suppresses the temperature-sensitivity caused by *sed4* deletion, while combination of *sar1-5* and *sed4-1* causes a strong transport block. These multiple genetic interactions argue that the functions of Sec16p, Sed4p and Sar1p are closely linked.

How the interactions of these proteins are coupled to vesicle morphogenesis can be inferred from what we know of their location with respect to the forming vesicle. Three classes of proteins that participate in vesicle budding are defined by the dissection of the membrane and cytosolic requirements for the reconstituted budding reaction and by localization experiments based on cell fractionation and immunofluorescence. The first class is associated with the ER membrane, but is not incorporated into vesicles, and

therefore probably functions in the ER membrane before completion of the vesicle. Representatives of this class are Sec12p (Rexach and Schekman, 1991; Barlowe et al., 1994) and Sed4p, as shown here. The second class, represented by the COPII proteins Sec13p/Sec31p, Sec23p/Sec24p and Sar1p (Barlowe et al., 1994) can be recruited from the cytosol to form a coat on the budded vesicles. In the accompanying paper, we show that Sec16p represents a third class of vesicle forming proteins that is tightly associated with the ER and is also incorporated into the vesicle coat.

From these localization studies and from the genetic interactions and binding studies we have developed a model for the function of Sed4p, Sec16p, Sec23p and Sar1p in the early steps of vesicle assembly (Figure 11). Because Sec16p is on both the ER and on vesicles it may serve as a scaffold for incorporation of soluble coat proteins into the vesicle. In the accompanying paper we show that the COPII protein, Sec23p, binds to the C-terminal domain of Sec16p. The genetic interactions between *SEC16* and *SAR1* are consistent with Sec16p also being a binding site for Sar1p. This proposed association of Sar1p with a complex of Sec16p and Sec23p is further supported by the observation that Sec23p stimulates Sar1p GTPase activity (Yosihisha et al., 1993). The function of Sed4p may be to promote the assembly or increase the stability of a nascent vesicle coat complex that includes Sec16p, Sec23p and Sar1p. This would explain why deletion of *SED4* exacerbates the transport defect of *sar1-5*, *sec16* and *sec23* mutations, and is consistent with Sed4p being a non-essential protein.

A specific function for *SED4*, suggested by sequence similarity to *SEC12*, would be to stimulate guanine-nucleotide exchange on Sar1p. The N-terminal domain of Sec12p has been shown to have such activity (Barlowe and Schekman, 1993), but parallel experiments using the partially purified N-terminal

domain of Sed4p did not show Sar1p-specific nucleotide exchange activity (C. Barlowe, personal communication). Although Sed4p does not have guanine-nucleotide exchange activity by itself, the complex between Sed4p and Sec16p may have this activity. To explore this possibility, we tested the soluble complex between the N-terminal domain of Sed4p and C-terminal domain of Sec16p for the ability to stimulate exchange of GTP for GDP by Sar1p. The complex was not active, but the truncations of Sed4p and Sec16p used to produce a soluble complex could have disrupted the capacity to associate with Sar1p. A more direct biochemical test of the interaction of Sar1p with Sec16p and Sed4p will depend on our ability to extract from membranes an active complex of these proteins.

Our data, together with the homology between Sed4p and Sec12p, suggest a role for Sed4p in the recruitment of Sar1p to a vesicle formation complex. Sec12p has been proposed to act similarly in the initial phases of vesicle formation by recruiting Sar1p to the membrane (d'Enfert et al., 1991b). However, our tests for functional overlap between *SED4* and *SEC12* show that these genes perform different functions. Increased dosage of *SED4* does not suppress *sec12* mutations and increased dosage of *SEC12* does not suppress the temperature-sensitivity of *sed4* deletions. Moreover, increased dosage of *SED4* suppresses *sec16* mutations, but parallel tests show no effect of increased dosage of *SEC12* on *sec16*. The biochemical properties of the N-terminal domains of Sec12p and Sed4p are also different: the N-terminal domain of Sed4p binds to the C-terminal domain of Sec16p, while no binding was detected using the corresponding domain of Sec12p.

One way to reconcile the apparently contradictory aspects of the relationship between *SED4* and *SEC12* would be to postulate that *SAR1* becomes engaged in vesicle formation through two functionally independent

pathways: one mediated by *SEC12* and the other mediated by *SED4* and *SEC16*. A genetic test of this idea would be to identify mutations in *SAR1* that affect one pathway but not the other. The *sar1-5* mutation appears to have this property as shown by tests for dosage-dependent suppression of *sec12* and *sec16* mutations. Increased dosage of *sar1-5* does not suppress *sec16-2* indicating that the mutation diminishes the effectiveness of Sar1p to function with Sec16p. However, increased dosage of *sar1-5* does suppress *sec12* mutations as effectively as wild type *SAR1,* showing no negative effect of *sar1-5* on the interaction of Sar1p with Sec12p. Moreover, *sar1-5* exacerbates *sec16* mutations, but has no effect on *sec12* mutations, further supporting the idea that *SAR1* engages in two independent processes and that *sar1-5* selectively disrupts the processes that involve *SEC16*. The two ways that *SAR1* functions in vesicle formation as distinguished by the *sar1-5* mutation are outlined in Figure 11.

The view suggested by our work is that Sec16p and Sed4p together may constitute a docking site needed to recruit Sar1p and coat proteins such as Sec23p to a nascent vesicle. Models for the formation of other coated vesicles have a similar outline. The binding of coatomer in formation of intra-Golgi transport vesicles and of AP-1 in formation of clathrin-coated vesicles have both been shown to depend on the action of the small GTP-binding protein, ADP-ribosylation factor (ARF) (Donaldson et al., 1992; Helms et al., 1992; Stamnes and Rothman, 1993; Traub et al., 1993). These same studies showed that assembly of both types of vesicle also requires Golgi membrane factors which presumably act as docking proteins for both ARF and coat subunits. The putative docking proteins for the Golgi have not yet been identified. We propose that Sed4p and Sec16p carry out this function at the ER membrane.

Knowledge of the interactions between *SEC16*, *SED4*, *SAR1* and *SEC23* offers a way to study the subunit associations in the early steps of ER vesicle

assembly free of the inherent biochemical complexity of the membrane. If soluble Sec16p can be obtained in an active form either as a recombinant protein or by extraction from membranes, it should be possible to develop assays in solution for the subunit assembly steps delineated here.

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Figure 1. *SED4* overexpression suppresses the temperature-sensitivity of *sec16-2*. Lanes 1 and 2: A *sec16-2* strain (CKY50) containing vector (pRS306-2μ) or *SED4* on a 2μ plasmid (pRH46). Lanes 3 and 4: A wild type strain (CKY8) containing vector (pRS306-2μ) or *SED4* on a 2μ plasmid (pRH46). Cells were spotted on selective medium and incubated at the indicated temperatures for 40h.

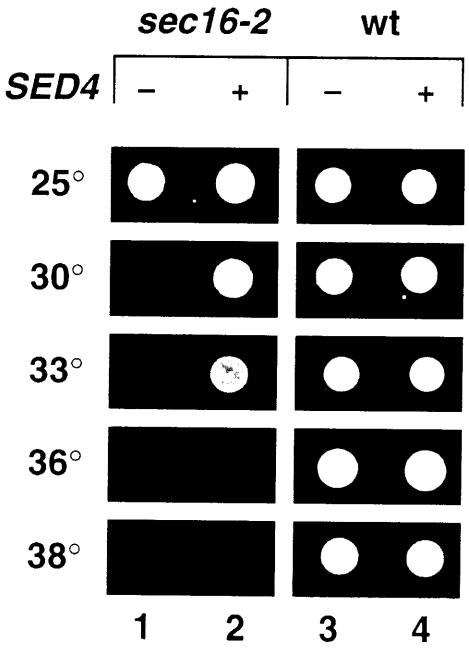

sec16-2
wt
SED4
–
+
–
+
25°
30°
33°
36°
38°
1
2
3
4

Figure 2. *SED4* overexpression suppresses the temperature-sensitive ER to Golgi transport defect of *sec16-2*. Lanes 1-5: A *sec16-2* strain (CKY50) carrying vector (pRS306-2μ). Lanes 6-10: A *sec16-2* strain carrying *SED4* on a 2μ plasmid (pRH46). Cells were grown in selective medium at 25°C, shifted to 32°C for 2h and pulse-labeled with [^{35}S]-methionine for 5 min. The label was chased for the times indicated. CPY was immunoprecipitated from extracts, resolved by SDS-PAGE, and imaged on a phosphorimager.

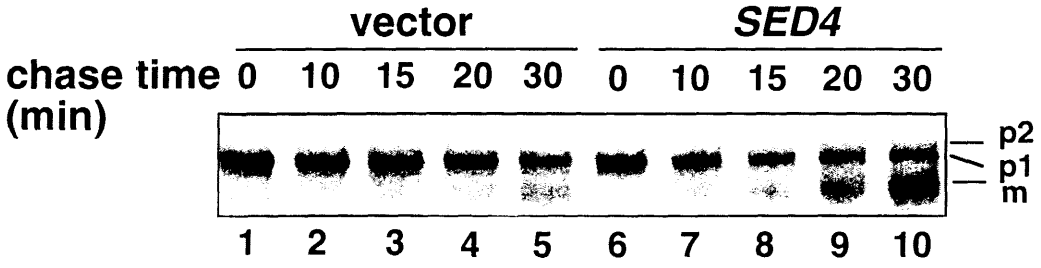

vector
SED4
chase time (min)
0 10 15 20 30 0 10 15 20 30
p2
p1
m
1 2 3 4 5 6 7 8 9 10

Figure 3. The N-terminal and transmembrane domains of *SED4* are necessary and sufficient for suppression of *sec16-2*. The indicated 2μ plasmids were transformed into CKY50 (*sec16-2*), CKY251 (*sed4-Δ1*), CKY39 (*sec12-4*) and RSY656 (*SEC12/sec12Δ*). Growth of single colonies assayed on selective medium at 33°C (*sec16-2, sec12-4*) or on rich medium at 41°C (*sed4-Δ1*) is shown. +++ indicates growth comparable to wild type, – indicates no growth. Complementation/suppression of *sec12-4* and *sec16-2* strains was also tested at 30° and 36°C with similar results. Suppression of *sec12Δ* was assayed by sporulating heterozygous *sec12Δ* diploids carrying test plasmids, dissecting 10 to 20 tetrads and scoring Leu^+ (*sec12Δ*) spores. +++ indicates that Leu^+ spores were readily obtained, – indicates that no Leu^+ spores were obtained.

			Suppression/Complementation			
			sec16-2	*sed4-Δ1*	*sec12-4*	*sec12Δ*
pRH50	*SEC12*		–	–	+++	+++
pRH46	*SED4*		+++	+++	–	–
pRH47	*sed4-T1*		+++	+++	–	n. d.
pRH54	*sed4-T2*		++	+++	–	n. d.
pRH78	*sed4-T3*		–	–	–	n. d.
pRH128	*sed4-T4*		–	–	–	n. d.
pRH62	*SEC12-SED4*		–	–	+++	+++
pRH141	*SEC12-SED4-HA*		–	–	+++	+++

Figure 4. **A.** Immunodetection of Sed4p-HA and comparison of the protein levels of Sed4p-HA and Sec12-Sed4p-HA fusion proteins. The *SEC12-SED4* fusion construct is described in Figure 3. Wild type cells (CKY10) carrying the indicated plasmids (pRH121, pRH120, pRH148, pRH141 and pRH46; lanes 1-5, respectively) were grown in selective medium. Extracts of 0.2 OD_{600} units of cells were resolved by SDS-PAGE on 6% gels and HA-tagged proteins were detected by Western blotting. **B.** Sed4p-HA is an O-glycosylated protein. Sed4p-HA was immunoprecipitated from extracts from either wild type (CKY10) (lanes 1, 2 and 5) or *dpm1-6* (PRY303) (lanes 3 and 4) strains carrying *SED4-HA* on a 2μ plasmid (pRH120). Cells were grown in minimal medium, shifted to the indicated temperatures for 15 min, and radiolabeled with [^{35}S]-methionine for 15 min. Tunicamycin was added to 10 μg/ml 5 min before labeling (lane 5). HA-tagged proteins were immunoprecipitated from extracts from 1 OD_{600} unit of cells and labeled proteins were visualized by fluorography after SDS-PAGE on a 6% gel.

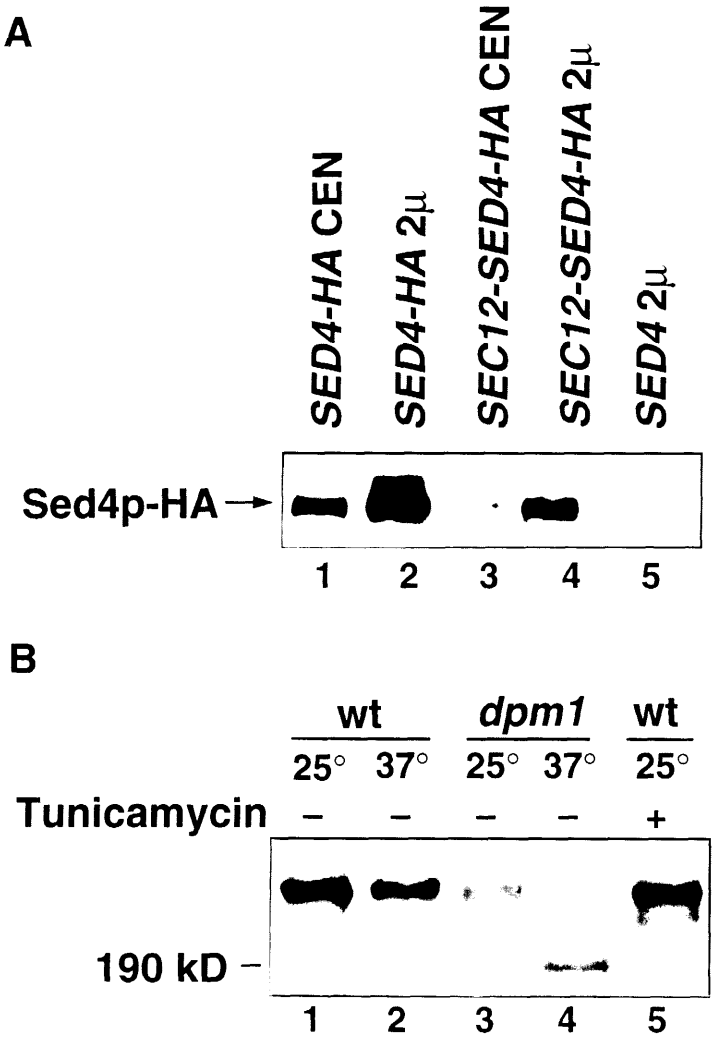

A
SED4-HA CEN
SED4-HA 2μ
SEC12-SED4-HA CEN
SEC12-SED4-HA 2μ
SED4 2μ
Sed4p-HA
1 2 3 4 5
B
wt
dpm1
wt
25° 37° 25° 37° 25°
Tunicamycin – – – – +
190 kD
1 2 3 4 5

Figure 5. Sed4p-HA is located in the ER. Indirect immunofluorescence of a wild type diploid strain (CKY8 x CKY10) carrying *SED4-HA* on a 2μ plasmid (pRH120). Fixed cells were incubated with both anti-HA and anti-BiP antibodies. The anti-HA antibody was visualized with rhodamine-coupled secondary antibody (top panel), the anti-BiP antibody was visualized with FITC-coupled secondary antibody (bottom panel).

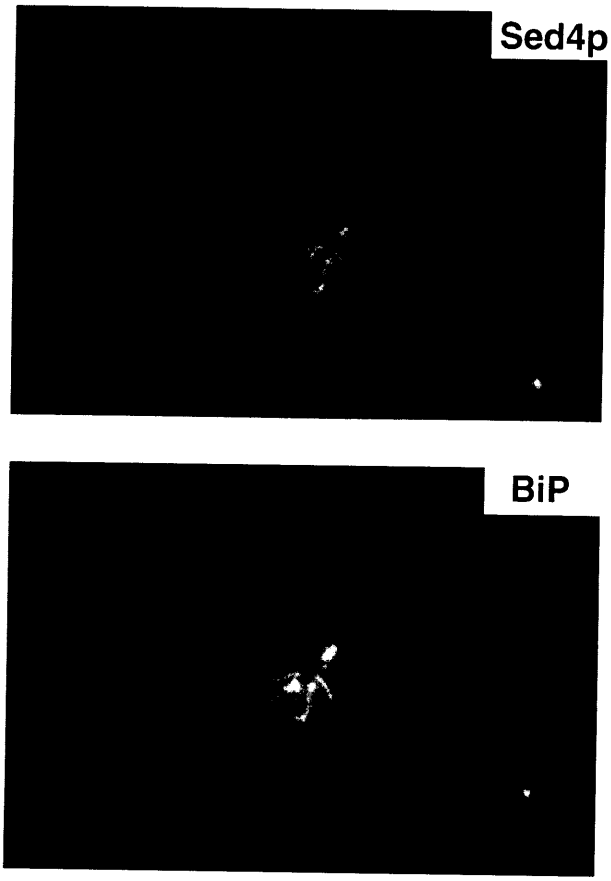

Sed4p
BiP

Figure 6. Sed4p-HA is not incorporated into ER-derived vesicles. Vesicles were produced *in vitro* using ER membranes from a *sed4-Δ1* strain containing *SED4-HA* on a low-copy plasmid (CKY295) (see Materials and Methods). The amount of Sed4p-HA or Sec22p in the starting membranes or the vesicle-containing high speed pellet (HSP) was determined by Western blotting and quantitated by densitometry. The ratio of protein in the HSP to protein in the starting membranes is expressed as % release.

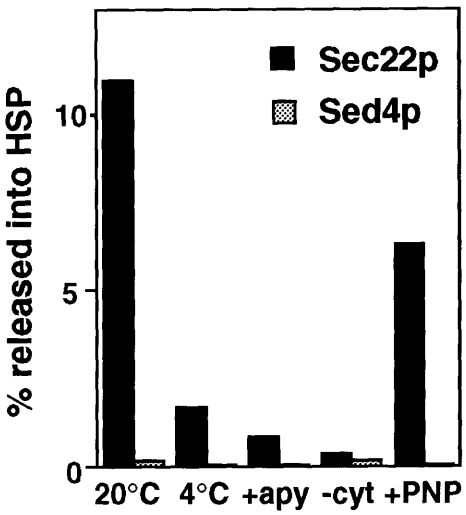

Sec22p
Sed4p
% released into HSP
10
5
0
20°C
4°C
+apy
-cyt
+PNP

Figure 7. The N-terminal domain of Sed4p and the C-terminal domain of Sec16p bind in extracts. The C-terminal domain of Sec16p fused to GST (pPE122, lanes 1 and 2) or GST only (pRD56, lanes 3 and 4) were expressed in yeast strain CKY289 together with either myc-tagged N-terminal domain of Sed4p (pRH260, upper row) or myc-tagged N-terminal domain of Sec12p (pRH261, lower row). Tagged proteins were detected by Western blotting after SDS-PAGE on a 8% gel. Lanes 1 and 3: Total extracts from 0.05 OD_{600} units of cells. Lanes 2 and 4: Material bound to glutathione beads from 0.4 OD_{600} units of cells.

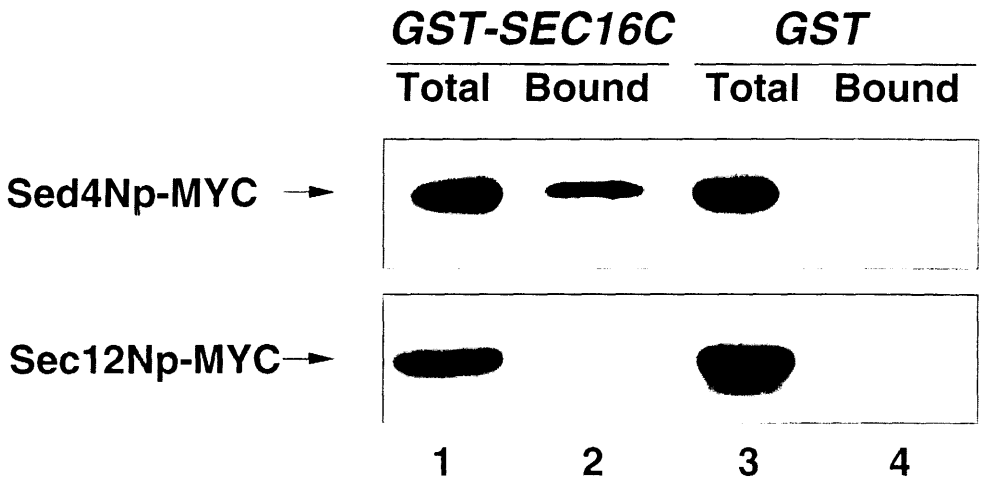
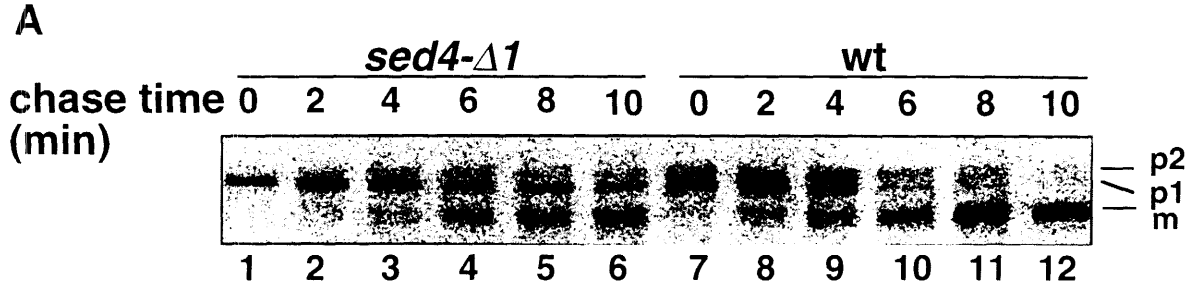

GST-SEC16C
GST
Total
Bound
Total
Bound
Sed4Np-MYC
Sec12Np-MYC
1
2
3
4

Figure 8. Deletion of *SED4* slows transport of CPY from the ER to the Golgi. **A.** Pulse-chase analysis of CPY in a *sed4-Δ1* strain (CKY251, lanes 1-6) or a wild type strain (CKY252, lanes 7-12). Cells grown at 38°C were pulse-labeled with [^{35}S]-methionine for 5 min. The label was chased for the times indicated. The different forms of CPY were immunoprecipitated from extracts and visualized on a phosphorimager after SDS-PAGE. **B.** Determination of kinetic parameters. The amount of radiolabeled p1 CPY and total CPY was quantitated for each time-point using the phosphorimager software. The ratio p1 CPY/total CPY gives the half-life of p1 from a linear curve-fit on a semilogarithmic plot using Cricket graph (v. 1.0). The half-life of p1 in a *sed4-Δ1* strain is 1.6-fold greater than wild type in the experiment shown. Similar results were obtained in four independent experiments.


A
sed4-Δ1
wt
chase time (min)
0 2 4 6 8 10 0 2 4 6 8 10
p2
p1
m
1 2 3 4 5 6 7 8 9 10 11 12

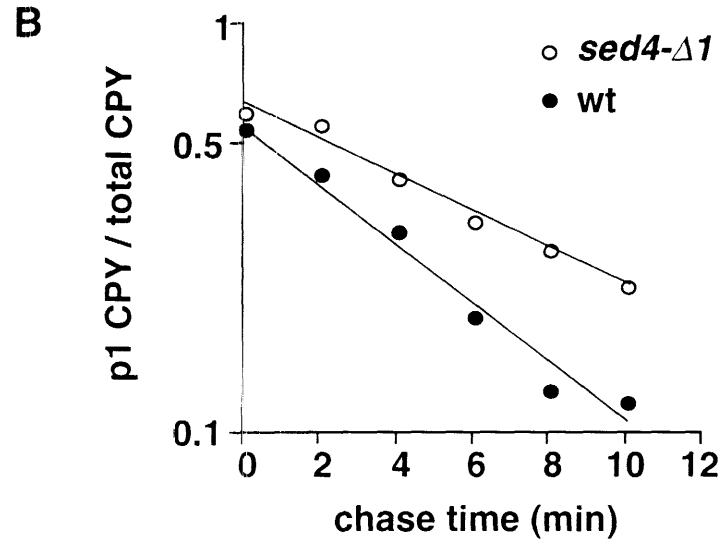

B
sed4-Δ1
wt
p1 CPY / total CPY
1
0.5
0.1
0 2 4 6 8 10 12
chase time (min)

Figure 9. Deletion of *SED4* in combination with a mutation in *SAR1* causes a temperature-sensitive growth and secretion defect. **A.** Wild-type (CKY291), *sed4-Δ1* (CKY292), *sar1-5 sed4-Δ1* (CKY293) and *sar1-5* (CKY294) cells were spotted on rich medium and incubated for 40h at 24°C or 38°C. **B.** CPY transport in the strains shown in A. Cells were grown in YPD at 30°C, shifted to 38°C for 2h and pulse-labeled with [^{35}S] methionine for 5 min. The label was chased for the times indicated and the different forms of CPY were immunoprecipitated from extracts, resolved by SDS-PAGE, and imaged on a phosphorimager.

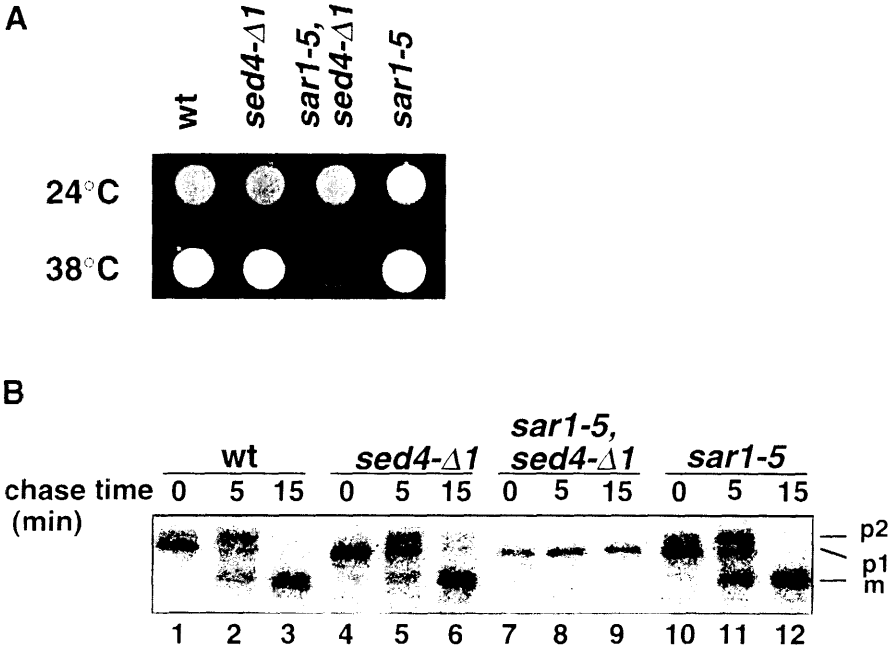

A
wt
sed4-Δ1
sar1-5, sed4-Δ1
sar1-5
24°C
38°C
B
wt
sed4-Δ1
sar1-5, sed4-Δ1
sar1-5
chase time (min)
0 5 15 0 5 15 0 5 15 0 5 15
p2
p1
m
1 2 3 4 5 6 7 8 9 10 11 12

Figure 10. A *sed4-Δ1 sar1-5* double mutant accumulates ER membranes, but not vesicles. Electron micrograph of *sed4-Δ1 sar1-5* (CKY293) grown at 25°C and shifted to 38°C for 2h. Membranes were stained with potassium permanganate. Arrows indicate excess ER. The bar represents 1 μm.

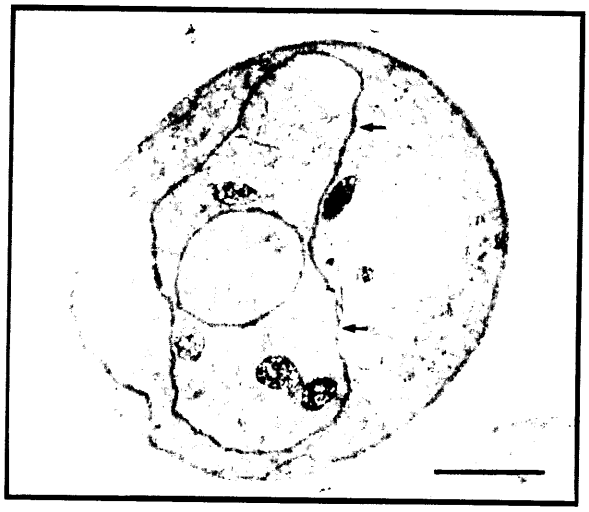


Figure 11. Proposed protein interactions early in vesicle formation. Interaction of Sar1p with Sec16p, Sec23p and Sed4p (1) is interrupted by *sar1-5*. Interaction of Sar1p with Sec12p (2) is not affected by *sar1-5*. Interaction of Sec16p with Sed4p and Sec23p is based on genetic interactions and binding studies.

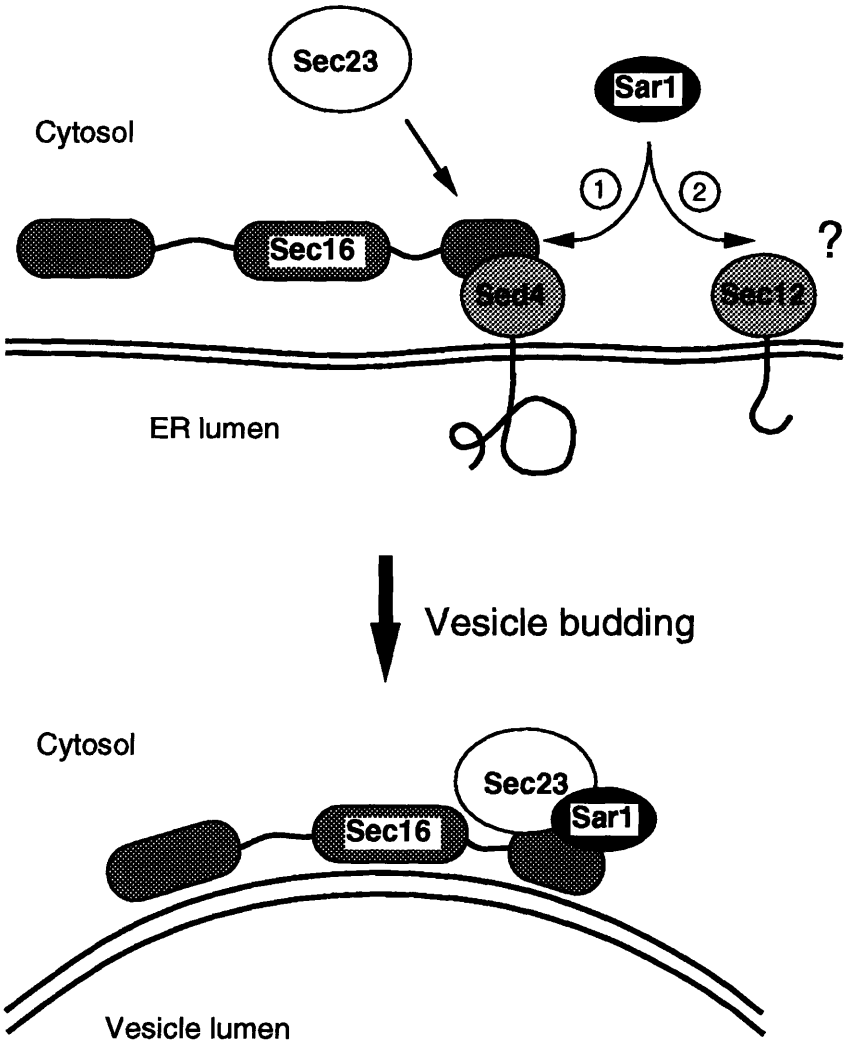

Sec23
Sar1
Cytosol
1
2
?
Sec16
Sed4
Sec12
ER lumen
Vesicle budding
Cytosol
Sec23
Sar1
Sec16
Vesicle lumen

Table I. *S. cerevisiae* Strains

Strain	Genotype	Source or Reference
CKY8	*MATα ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY10	*MATa ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY93	*MATα ura3-52 leu2 pep4::URA3*	Kaiser Lab Collection
CKY289	*MATa ura3-52 leu2 his3Δ200 trp1Δ63 lys2-801 Gal$^+$*	Kaiser Lab Collection
CKY249	*MATa/Matα SED4/sed4-Δ1::URA3 ura3-52/ura3-52 leu2-3,112/leu2-3,112*	This study
CKY250	*MATa/Matα SED4/sed4-Δ1 ura3-52/ura3-52 leu2-3,112/leu2-3,112*	This study
CKY251	*MATα sed4-Δ1 ura3-52 leu2-3,112*	This study
CKY252	*MATα ura3-52 leu2-3,112*	This study
CKY255	*MATα sed4-Δ1::URA3 ura3-52 leu2-3,112*	This study
CKY258	*MATa sed4-Δ1::URA3 ura3-52 leu2-3,112*	This study
CKY291	*MATα ura3-52 leu2-3,112*	This study
CKY292	*MATα sed4-Δ1::URA3 ura3-52 leu2-3,112*	This study
CKY293	*MATa sed4-Δ1::URA3 sar1-5 ura3-52 leu2-3,112*	This study
CKY294	*MATa sar1-5 ura3-52 leu2-3,112*	This study
CKY295	*MATα sed4-Δ1 ura3-52 leu2-3,112 pep4::LEU2* (pRH121)	This study
CKY296	*MATα sed4-Δ1 SAR1::URA3 ura3-52 leu2-3,112*	This study
CKY39	*MATα sec12-4 ura3-52 his4-619*	Kaiser Lab Collection
CKY45	*MATα sec13-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY50	*MATα sec16-2 ura3-52 his4-619*	Kaiser Lab Collection
CKY52	*MATa sec16-1 ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY54	*MATα sec17-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY58	*MATα sec18-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY62	*MATα sec19-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY64	*MATα sec20-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY69	*MATa sec21-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY70	*MATα sec22-3 ura3-52 his4-619*	Kaiser Lab Collection
CKY78	*MATa sec23-1 ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY105	*MATa sec16-3 ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY230	*MATα sec16-4 ura3-52 leu2-3,112 ade2 ade3 sec13-1 (pCEN-ADE3-SEC13)*	Kaiser Lab Collection
NY768	*MATα sec1-1 ura3-52 leu2-3,112*	P. Novick (Yale University)
NY770	*MATα sec2-41 ura3-52 leu2-3,112*	P. Novick (Yale University)
NY772	*MATa sec3-52 ura3-52 leu2-3,112*	P. Novick (Yale University)
NY774	*MATα sec4-8 ura3-52 leu2-3,112*	P. Novick (Yale University)
NY776	*MATα sec5-24 ura3-52 leu2-3,112*	P. Novick (Yale University)
NY778	*MATα sec6-4 ura3-52 leu2-3,112*	P. Novick (Yale University)
NY780	*MATα sec8-9 ura3-52 leu2-3,112*	P. Novick (Yale University)
NY782	*MATa sec9-4 ura3-52 leu2-3,112*	P. Novick (Yale University)
NY784	*MATa sec10-2 ura3-52 leu2-3,112*	P. Novick (Yale University)
NY786	*MATa sec15-1 ura3-52 leu2-3,112*	P. Novick (Yale University)
AFY72	*MATa sec7-1 ura3-1 his3-11 trp1-1*	R. Schekman (U.C. Berkeley)
ANY123	*MATα bet1-1 ura3-52 his4-619*	S. Ferro-Novick (Yale University)
ANY125	*MATα bet2-1 ura3-52 his4-619*	S. Ferro-Novick (Yale University)
RSY533	*MATα sec61-2 ura3-52 leu2-3,112 ade2 pep4-3*	R. Schekman (U.C. Berkeley)
RSY530	*MATα sec62 ura3-52 leu2-3,112*	R. Schekman (U.C. Berkeley)
RSY153	*MATα sec63-1 ura3-52 leu2-3,112*	R. Schekman (U.C. Berkeley)
CKY234	*MATα sec16::TRP1 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 ura3-52 leu2-Δ2* (pPE5)	Espenshade et al., 1995
RSY656	*MATa/Matα SEC12/sec12Δ::LEU2 ura3-1/ura3-1 leu2-3/leu2-3 trp1-1/trp1-1 ade2-1/ade2-1 his3-11/his3-11 can1-100/can1-100*	d'Enfert et al., 1991a
EGY40	*MATα ura3-52 leu2 his3 trp1*	Golemis and Brent, 1992
PRY303	*MATα dpm1::LEU2 leu2-3,112 lys2-801 trp1Δ1 ura3-52* (p*dpm1-6*)	Orlean, 1990

Table II. Plasmids

Plasmid	Description	Source or Reference
pRS306	integrating vector marked with *URA3*	Sikorski and Hieter, 1989
pRS316	centromere vector marked with *URA3*	Sikorski and Hieter, 1989
pRS313	centromere vector marked with *HIS3*	Sikorski and Hieter, 1989
pRS315	centromere vector marked with *LEU2*	Sikorski and Hieter, 1989
pRS306-2μ	2μ vector marked with *URA3* (pRS306 derivative)	Miller and Fink, unpublished
pRS305-2μ	2μ vector marked with *LEU2* (pRS305 derivative)	Miller and Fink, unpublished
pRH46	*SED4* in pRS306-2μ	This study
pRH107	*SED4* in pRS316	This study
pPE87	*SED4* in pRS305-2μ	This study
pRH120	*SED4-HA* in pRS306-2μ	This study
pRH121	*SED4-HA* in pRS316	This study
pRH47	*SED4* (1-841) in pRS306-2μ	This study
pRH54	*SED4* (1-369) in pRS306-2μ	This study
pRH78	*SED4* (1-343) in pRS306-2μ	This study
pRH128	*SED4* (1-294/331-1061) in pRS 306-2μ	This study
pRH62	*SEC12* (1-374) fused to *SED4* (368-1065) in pRS306-2μ	This study
pRH141	*SEC12* (1-374) fused to *SED4-HA* (368-1065) in pRS306-2μ	This study
pRH148	*SEC12* (1-374) fused to *SED4-HA* (368-1065) in pRS316	This study
pRH50	*SEC12* in pRS306-2μ	This study
pRH213	*SEC12* in pRS316	This study
pPE5	*SEC16* in YCp50	Espenshade et al., 1995
pPE8	*SEC16* in pRS315	Espenshade et al., 1995
pKR1	*SEC13* in pRS316	Roberg and Kaiser, unpublished
pCK1313	*SEC13* in YEp352	Pryer et al., 1993
YCP1142	*SEC23* in YCp50	Hicke and Schekman,1989
pRH259	*SAR1* in pRS316	This study
pRH262	*sar1-5* in pRS316	This study
pRH279	*sar1-5* in pRS306-2μ	This study
pRH280	*SAR1* in pRS306-2μ	This study
pEG202	*lexA* DNA binding domain in a 2μ vector marked with *HIS3*	Gyuris et al., 1993
pJG4-5	acidic activation domain in a 2μ vector marked with *TRP1*	Gyuris 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
pPE58	*SEC16* (1645-2194) in pEG202	Espenshade et al., 1995
pPE59	*SEC16* (1-824) in pEG202	Espenshade et al., 1995
pPE74	*SEC16* (447-1737) in pEG202	Espenshade et al., 1995
pRH151	*SED4* (1-347) in pJG4-5	This study
pRH152	*SEC12* (1-354) in pJG4-5	This study
pRH260	*GAL10* promoted *SED4-MYC* (1-347) in pRS313	This study
pRH261	*GAL10* promoted *SEC12-MYC* (1-354) in pRS313	This study
pRD56	*GAL1* promoted *GST* in pRS316	R. Deshaies
pPE122	*GAL1* promoted *GST-SEC16* (1638-2194) in pRS316	This study

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

Table III. The N-terminal Domain of Sed4p and the C-terminal Domain of Sec16p Interact in the Two-Hybrid Assay

lexA DNA-binding domain	Activation domain ß-Gal (units)		
	SED4N	*SEC12N*	no fusion
*SEC16*C	681.6 ± 77.2	21.6 ± 0.3	18.9 ± 0.1
*SEC16*N	18.6 ± 0.9	15.7 ± 2.8	16.1 ± 2.1
*SEC16*CEN	18.2 ± 2.0	17.2 ± 0.0	22.5 ± 1.1
no fusion	100.3 ± 4.5	146.3 ± 65.4	74.1 ± 7.2

Interactions were assayed for two independent transformants as described in Materials and Methods. The values given are means ± SD. Plasmids used were pPE58 (*SEC16C*), pPE59 (*SEC16N*), pPE74 (*SEC16CEN*), pRH151 (*SED4N*) and pRH152 (*SEC12N*).

Table IV. Deletion of *SED4* Exacerbates the Growth Defect of Mutants Defective in Vesicle Formation

Genotype	Incubation Temperature		
	28°	30°	33°
A. Vesicle formation:			
sec12-4	+++	+	–
sec12-4 sed4-Δ1::URA3	±	–	–
sec13-1	+++	++	–
sec13-1 sed4-Δ1::URA3	+	±	–
sec16-2	+++	±	–
sec16-2 sed4-Δ1::URA3	±	–	–
sec23-1	+++	–	–
sec23-1 sed4-Δ1::URA3	–	–	–
B. Vesicle fusion:			
sec17-1	+++	+++	±
sec17-1 sed4-Δ1::URA3	+++	+++	–
sec18-1	+	–	–
sec18-1 sed4-Δ1::URA3	+	–	–

Growth of single colonies on YPD after 24 to 48h. +++ is growth comparable to wild-type, – is no growth.

Table V. Genetic Interactions of *sar1-5* with Vesicle Formation Mutants

Genotype	Incubation Temperature				
	24°	27°	30°	33°	38°
sec12-4	+++	+++	+	–	–
sec12-4 sar1-5	+++	+++	+	–	–
sec12-4 (p*SAR1*)	+++	+++	+++	+++	+++
sec12-4 (p*sar1-5*)	+++	+++	+++	+++	+++
sec13-1	+++	+++	++	–	–
sec13-1 sar1-5	+++	±	–	–	–
sec16-2	+++	+++	±	–	–
sec16-2 sar1-5	+++	±	–	–	–
sec16-2 (p*SAR1*)	+++	+++	++	++	–
sec16-2 (p*sar1-5*)	+++	+++	±	–	–
sec23-1	+++	+++	–	–	–
sec23-1 sar1-5	++	++	–	–	–
sec23-1 (p*SAR1*)	+++	+++	++	–	–
sec23-1 (p*sar1-5*)	+++	+++	–	–	–

p*SAR1* is pRH259 or pRH280. p*sar1-5* is pRH262 or pRH279. Growth of single colonies on YPD after 24 to 48h. +++ is growth comparable to wild-type, – is no growth.

Chapter 4

Iss1p: a Sec24p Homolog that Binds Sec16p and Sec23p, and Facilitates Docking of Endoplasmic Reticulum Derived Vesicles

PREFACE

This chapter represents primarily my own work. Alison Frand isolated the *sec24-1* mutant and performed some of the genetic crosses presented in Table III.

ABSTRACT

Secretory proteins are transported from the endoplasmic reticulum (ER) to the Golgi apparatus in membrane-bounded vesicles that are surrounded by a proteinaceous coat, termed COPII. Sec24p is one of six proteins that are required for formation of COPII coated vesicles, and that are themselves components of the COPII coat. Sec24p associates with two additional COPII coat components, Sec23p and Sec16p, and a Sec23p/Sec24p/Sec16p complex has been suggested to be an important intermediate during COPII coat assembly. We identified a Sec24p homolog, Iss1p, in a screen for proteins that bind to the central domain of Sec16p. Iss1p, like Sec24p, binds to Sec16p and to Sec23p, as demonstrated by two-hybrid and coprecipitation assays. Iss1p can functionally substitute for Sec24p, since overexpression of *ISS1* rescues the growth and secretion defect of a temperature-sensitive *sec24* mutant. However, genetic interactions of *ISS1* suggest, that Iss1p acts to facilitate docking, rather than formation, of COPII coated vesicles. *iss1* deletion exacerbates mutations defective in vesicle docking, but not those that affect formation of ER-derived vesicles. Furthermore, *ISS1* overexpression suppresses mutations in the vesicle docking gene *SEC22*.

A temperature-sensitive *sec24* mutant exacerbates mutants defective in vesicle formation at the ER, consistent with the known role of Sec24p in this process. In contrast to other mutants in COPII coat components, however, a *sec24* mutant also exacerbates mutants defective vesicle docking. We suggest that Sec24p, in addition to its function in vesicle formation, facilitates docking of ER-derived vesicles.

INTRODUCTION

COPII coated vesicles transport secretory proteins 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 identified six proteins that are required for the assembly of COPII coated vesicles and are themselves components of the COPII coat (Novick et al., 1981; Barlowe et al., 1994; Espemshade et al., 1995). Five of these proteins - two heteromeric protein complexes, Sec23p/Sec24p and Sec13p/Sec31p, and the small GTP-binding protein Sar1p - are present in the cytosol and are recruited to the ER membrane during vesicle formation in vitro (Salama et al., 1993; Barlowe et al., 1994). A sixth protein, Sec16p, is tightly associated with ER membranes and has been suggested to act as a scaffold onto which cytosolic COPII coat components assemble (Espenshade et al., 1995; Gimeno et al., in press). Assembly of the COPII coat is thought to be regulated by the small GTP-binding protein Sar1p. In its GTP-bound form, Sar1p promotes COPII coat formation, while GTP hydrolysis on Sar1p is required for coat disassembly (Barlowe et al., 1994; Oka and Nakano, 1994).

Formation of COPII coated vesicles is coupled to sorting of cargo proteins. One class of cargo proteins are integral ER membrane proteins that are required for vesicle docking, such as Sec22p, Bos1p and Bet1p (Rexach et al., 1994; Barlowe et al., 1994). Sec22p and Bos1p form a docking complex with proteins at the acceptor membrane; formation of this complex is required for vesicle fusion to proceed (Rothman, 1994). A small GTP-binding protein, Ypt1p, activates Sec22p and Bos1p, and is required for formation of the docking complex (Lian et al., 1994; Soogard et al., 1994). Since vesicles formed in vitro in the presence of a non-hydrolyzable GTP analog are blocked in docking prior to the requirement

for Ypt1p, it is thought that COPII coat disassembly precedes Sec22p/Bos1p activation and vesicle docking (Oka and Nakano, 1994).

The function of the COPII coat component Sec24p is still largely unclear. Sec24p is a 105 kD protein that is present in the cytosol in a 300-400 kD complex with Sec23p. Both Sec23p and Sec24p are required for vesicle formation in vitro (Hicke et al., 1992), and a *sec23* mutant has a vesicle formation defect in vivo (Kaiser and Schekman, 1990). Furthermore, both Sec23p and Sec24p bind to Sec16p, and it has been suggested that a Sec23p/Sec24p/Sec16p complex is an intermediate in COPII vesicle formation (Gimeno et al., in press). Although Sec23p has GAP activity with respect to Sar1p, Sec24p does not modulate this activity of Sec23p (Yoshihisa et al., 1993).

We identified a Sec24p homolog, Iss1p, in a two-hybrid screen for proteins that interact with the central domain of Sec16p. Iss1p is similar to Sec24p in sequence, protein-protein interactions and some genetic interactions, suggesting that Iss1p functions in a manner similar to Sec24p. Surprisingly, we found that *ISS1* and *SEC24*, but not other COPII coat component genes, have genetic interactions indicative of a role in vesicle docking. We suggest that Sec24p and its homolog, Iss1p, function to ensure efficient docking of ER-derived transport vesicles.

MATERIALS AND METHODS

Strains, Media and General Techniques. Yeast media were prepared and yeast genetic and molecular biological techniques were performed using standard methods (Kaiser et al., 1994) All experiments on plasmid-bearing strains were performed on at least two independent transformants. DNA manipulations were carried out using standard methods (Sambrook et al., 1989). DNA sequencing was performed using the Sequenase kit (USB, Cleveland, OH). 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-CPY (1/1,000), anti-Sec23p (1/500; kindly provided by L. Hicke) and HRP-coupled sheep anti rabbit Ig (1/10,000; Amersham Corp., Arlington Heights, IL). DNA sequence comparisons were performed using the BestFit program (Genetics Computer Group).

Isolation and Two-Hybrid Analysis of *ISS1*. *ISS1* was isolated in a two-hyrid screen for proteins that bind to the central domain of Sec16p. The screen was performed using EGY48 carrying the plasmid pSH18-34 as an indicator strain (Golemis et al., 1994). In this strain, positive interactions can be detected as growth on medium lacking leucine - reflecting expression of a *LEU2* reporter gene in EGY48 - and by measuring the expression of β-galactosidase from the reporter plasmid pSH18-34. A plasmid containing the central domain of Sec16p (amino acids 447-1235) fused to the *lexA* DNA binding domain (pPE62) was used as a bait (Gimeno et al., in press), and a *S. cerevisiae* genomic library fused to an acidic activation domain in the vector pJG4-5 was used as a fish (Watt et al., 1995). ~2 x 10^5 transformants were replica-plated onto SC medium lacking leucine and supplemented with 2% galactose. Colonies able to grow were tested for expression of β-galactosidase by patching on SC medium (pH 7.0) lacking the

appropriate amino acids and supplemented with 2% galactose and 40 mg/l X-gal. Library plasmids were recovered from Leu$^+$, blue colonies. Two sets of plasmids, representing two distinct genes were isolated. These genes were named *ISS1* and *ISS2* (Interacting with Sec sixteen 1 and 2). DNA sequencing of the fusion junction and comparison to the database showed that the library clone of *ISS1* (pRH231) contained amino acids 597-875 of Iss1p fused to the acidic activation domain.

Two full-length clones of *ISS1* (pRH194 and pRH195) were isolated from a library of yeast genomic DNA in pCT3 (Thompson et al., 1993) by hybridization using the 1 kb EcoRI fragment of pRH231 as a probe. A subclone containing the entire *ISS1* reading frame flanked by 2.5 kb and 2 kb genomic DNA at the 5' and 3' end (pRH200), was made by cutting pRH194 with *Xba*I and ligating to itself. To express *ISS1* on a multicopy vector, the 8 kb *BamH*I-*Cla*I fragment of pRH195 was cloned into pRS306-2μ, creating pRH210. pRH210 was then cut with *Sac*I and ligated to itself, creating pRH310. pRH310 contains the entire *ISS1* reading frame flanked by 2.5 kb and 1 kb genomic DNA.

The protein-protein interactions of Iss1p were tested in vivo using the two-hybrid system. Plasmids were constructed as follows: To make a fusion of the entire *ISS1* sequence to the *lexA* DNA-binding domain (pRH266), the N-terminus of *ISS1* was amplified by PCR and ligated into pCD43, a pRS316 derivative. The C-terminus of *ISS1* was then added by inserting the 2.8 kb *Bgl*II fragment of pRH200 into the resulting construct, creating pRH256. pRH266 was made by inserting the 3 kb *BamH*I-*Not*I fragment of pRH256 into pEG202. The N-terminal *ISS1* fusion to *lexA* (pRH319) contains the 1.9 kb *EcoR*I fragment of pRH266 ligated into pEG202. The fusion of the C-terminal region of *ISS1* to *lexA* (pRH249) was made by inserting the 1.2 kb *EcoR*I fragment of pRH321 into

pEG202-AAT, a derivative of pEG202 that has a different reading frame in the polylinker.

All interactions were assayed in EGY40 carrying the indicator plasmid pSH18-34. To measure interactions between Iss1p and Sec16p, indicator cells were grown to exponential phase in selective medium containing 2% glucose. To measure interactions between Sec23p and Sec16p or Iss1p, 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 3 h. β-galactosidase activity assays were performed 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. *ISS1* (aa1-875; pRH303) was fused to GST by inserting the 3 kb *BamH*I-*Xho*I fragment of pRH266 into pPE128, a derivative of pRD56 that has a different reading frame in the polylinker. 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. To facilitate detection of Sec23p, *SEC23* was expressed from the *GAL1* promoter (pPE123).

Coprecipitation experiments were performed in CKY473 as described (Gimeno et al., in press).

Construction of an *iss1* Deletion Allele. A chromosomal deletion *iss1-Δ1::TRP* that replaced amino acids 116-622 of *ISS1* with the *TRP1* marker was made using a one-step gene disruption technique (Rothstein, 1991). The disruption plasmid (pRH247) was constructed as follows. A *Spe*I fragment of pRH200 was cloned into pRS306 creating pRH217. After deleting the *EcoR*I site from the

polylinker, the 1.5 kb *Bgl*II-*EcoR*I fragment of pRH217 was replaced with a 1 kb fragment containing the *TRP1* marker, creating pRH247.

A *trp1* diploid, CKY19, was transformed with the 2.3 kb *Spe*I fragment of pRH347, yielding CKY498. Tetrad analysis of CKY498 gave 2:2 segregation of *TRP1*. Integration of *TRP1* at the *ISS1* locus was confirmed by Southern blotting (ECL kit, Amersham).

RESULTS

Iss1p is Homologous to Sec24p.

To identify proteins that bind to the central domain of Sec16p (amino acids 447-1235), we screened a library of *S. cerevisiae* genomic DNA fused to an acidic activation domain for plasmids that confer interaction with the central domain of Sec16p fused to the *lexA* DNA binding domain. Positive interactions bring together the *lexA* DNA binding domain and the acidic activation domain to drive transcription of *LEU2* and *lacZ* reporter genes, and were detected by their ability to confer growth on medium lacking leucine and blue color on medium containing X-GAL. Two sets of plasmids interacted strongly with the central domain of Sec16p; the corresponding genes were named *ISS1* and *ISS2* (Interacting with Sec Sixteen 1 and 2). DNA sequencing and database comparison showed that *ISS1* encodes a 99 kD protein with strong similarity to the COPII coat component Sec24p (Fig. 1 A), while *ISS2* encodes a novel protein. *ISS2* will be described elsewhere.

The protein sequence of Iss1p is 62% identical to Sec24p (Fig. 1 A). This sequence similarity extends throughout the length of the protein, and defines two variable regions in the N-terminal part of the proteins, a glutamine-rich domain (amino acids 17-143 in Sec24p, 17-67 in Iss1p) and a charged domain (amino acids 362-372 in Sec24p, 295-327 in Iss1p). We noticed that both Iss1p and Sec24p contain a putative Zn-binding motif (CX_2C-CX_2C) near their N-terminal domains (Fig. 1 A, bold amino acids; Fig. 1B). Similar Zn-binding motifs have been found in several proteins that direct secretory pathway function, such as a GTPase activating protein for ARF1 (ARF-GAP), and in two genes required for transport of proteins to the vacuole, Vps11p and Vps18p; these Zn-binding motifs are important for the function of at least ARF-GAP and Vps18p (Robinson et al., 1991, Cukierman et al., 1995). Interestingly, Vps11p and Vps18p, two

proteins that are likely to function at a common step in the secretory pathway, share sequence homology in the region of the Zn-binding motif (Robinson et al., 1991). Database searches with the putative Zn-binding domain of Sec24p showed that Sec23p contains a homologous Zn-binding motif in its N-terminal domain (Fig. 1 B), suggesting that Sec24p and Sec23p belong to a group of proteins that are functionally related and share a homologous Zn-binding motif. It will be interesting to determine whether the Zn-binding motif is important for the function of Sec23p and/or Sec24p, and if so, what effects it has on the protein-protein interactions of Sec23p and Sec24p. Our database search also identified a novel protein, YHR098c, that shares homology with Sec24p in the Zn-binding domain (Fig. 1 B), but also shows weak sequence similarity with Sec24p throughout the length of the protein (21%). YHR098c is likely a more distant Sec24p homolog.

Iss1p Binds to the Central Domain of Sec16p and to Sec23p.

The strong sequence similarity between Iss1p and Sec24p suggested that Iss1p, like Sec24p, may bind Sec23p and the central domain of Sec16p. Indeed, full-length Iss1p interacted with both the central domain of Sec16p (amino acids 565-1235) and with Sec23p in the two-hybrid assay (Fig. 2 A). We had previously demonstrated that Sec23p and Sec16p bind to distinct domains on Sec24p: the central domain of Sec16p binds to both the N-terminal and the C-terminal domain of Sec24p, while Sec23p binds to the N-terminal domain of Sec24p only (Gimeno et al., in press). To determine whether Iss1p interacted with Sec16p and Sec23p in a similar manner, we constructed fusions corresponding to the N-terminal and C-terminal portions of Iss1p. The C-terminal domain of Iss1p interacted with the central domain of Sec16p only, while the N-terminal domain of Iss1p interacted only with Sec23p (Fig. 2 A). Since the

interaction between the N-terminal Iss1p fusion and Sec23p is weak, it is possible that this fusion protein is partially inactive, possibly explaining the lack of an interaction between the Iss1 N-terminus and the central domain of Sec16p. However, it is clear that Iss1p, like Sec24p, has distinct binding sites for Sec23p and the central domain of Sec16p.

We also used the two-hybrid system to examine the part of Sec16p with which the C-terminus of Iss1p interacts. The C-terminus of Iss1p (amino acids 622-875) fused to an acidic activation domain was tested for interactions with three Sec16p fragments fused to the *lexA* DNA-binding domain (Fig. 1B). Like the C-terminal domain of Sec24p (Gimeno et al., in press), the C-terminal domain of Iss1p interacted only with the central domain of Sec16p (Fig. 1 B). Thus, Iss1p is also similar to Sec24p in its interaction with Sec16p.

To confirm the interactions between Iss1p and Sec23p/Sec16p, we tested the ability of these proteins to associate in yeast extracts. Iss1p was fused to glutathione-S-transferase (GST) and expressed in yeast from the *GAL1* promoter. The central domain of Sec16p (amino acids 565-1235) fused to invertase or full-length Sec23p were 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 either GST-Iss1p or GST alone and either the Sec16p-invertase fusion protein or Sec23p. GST-Iss1p associated with both the central domain of Sec16p (Fig. 3, top panel, lanes 1 and 2) and with Sec23p (Fig. 3, bottom panel, lanes 1 and 2). No binding was detected for a control that used GST only (Fig. 3, lanes 3 and 4). These results confirm that Iss1p, like Sec24p, can form a complex with Sec23p and with the central domain of Sec16p.

Deletion of *iss1* Exacerbates Vesicle Docking Mutants.

Since Iss1p is similar to Sec24p in both sequence and protein-protein interactions, we were interested in whether Iss1p functions in ER to Golgi transport. A null allele of *ISS1* (*iss1-Δ1::TRP1*) was constructed by replacing amino acids 166-622 with the *TRP1* marker (see Materials and Methods). Strains deleted for *iss1* grew as well as wild-type strains at temperatures between 25° and 38°C, and had no obvious secretion defects (data not shown). In particular, *iss1-Δ1::TRP1* strains did not accumulate intermediate forms of CPY or invertase as judged by Western Blotting, and showed wild-type kinetics of CPY maturation in pulse-chase experiments at 30°C (data not shown). Furthermore, *iss1* deletion strains did not secrete resident ER proteins, such as Kar2p, or the soluble vacuolar protein CPY, suggesting that Iss1p is not required for retrograde transport from the Golgi apparatus to the ER.

To determine whether Iss1p functions in the secretory pathway and, if so, at which step, we examined synthetic lethal interactions between *iss1-Δ1::TRP1* and a panel of secretion mutants (Table III). Synthetic lethal interactions have been used extensively to define the step in the secretory pathway where a gene product acts, and so far have only been observed between genes that affect the same step of the secretory pathway (Salminen and Novick, 1987; Newman et al., 1987; Rothblatt et al., 1989; Kaiser and Schekman, 1990; Gimeno et al., 1995). Surprisingly, *iss1-Δ1::TRP1* did not affect mutants defective in vesicle formation (*sec12-4, sec13-1, sec16-2, sec23-1*) or vesicle fusion (*sec17-1, sec18-1*), but showed strong synthetic lethal interactions with mutants defective in docking of ER-derived vesicles (*sec22-3, bet1-1*; Table III). These data argue that Iss1p does not function during formation of vesicles at the ER, but is required for efficient docking of ER-derived vesicles with the Golgi apparatus.

Overexpression of *ISS1* Suppresses Mutations in the Vesicle Docking Gene *SEC22*.

We also examined the effects of *ISS1* overexpression on temperature-sensitive mutants in a panel of secretion genes. *ISS1* on a low or high copy plasmid was transformed into the *sec* and *bet* mutants listed in Table I and tested for growth at 30, 33, 36 and 38°C. *ISS1* overexpression suppressed the growth defect of both *sec22* alleles tested (Fig. 4 and data not shown), but had no effect on the growth of any of the other mutants, with the exception of *sec24-1* (see below). The ability of *ISS1* overexpression to suppress *sec22* is consistent with its genetic interactions with vesicle docking mutants, and strongly argues for a role for Iss1p in vesicle docking.

Overexpression of *ISS1* Suppresses a Temperature-sensitive *sec24* Mutant.

Since Iss1p is similar to Sec24p in both sequence and protein-protein interactions, we examine whether Iss1p could substitute for Sec24p during ER to Golgi transport. A temperature-sensitive allele of *SEC24*, *sec24-1*, was isolated from a collection of random Ts mutants by screening for mutants that accumulate the ER froms of invertase and carboxypeptidase Y (CPY) at the non-permissive temperature (E. Chitouras and C. Kaiser, unpublished; Gimeno et al., in press). The *sec24-1* mutant is unable to grow at temperatures above 27°C and accumulates the ER (p1) form of the secretory marker protein CPY at temperatures of 30°C or higher (Fig. 5 A and B). Expression of *ISS1* from a high copy (2μ) plasmid allowed growth of *sec24-1* at temperatures up to 36°C (Fig. 5 A), and considerably decreased the amount of p1 CPY accumulated at 30°C (Fig. 5 B, lanes 4-6). These data indicate that Iss1p can indeed functionally substitute for Sec24p.

A Temperature-sensitive *sec24* Mutant Exacerbates Vesicle Docking Mutants.

The genetic interactions of *ISS1* with vesicle docking genes prompted us to examine the synthetic lethal interactions of *sec24-1* in more detail. Since Sec24p is required for COPII vesicle formation in vitro (Hicke et al., 1992), we expected to find strong synthetic lethal interactions between *sec24* mutants and other vesicle formation mutants (*sec12-4, sec13-1, sec16-2, sec23-1*). This was indeed the case (Table III). In addition, however, we found strong synthetic lethal interactions between *sec24-1* and mutants defective in vesicle docking (*sec22-3, bet1-1*) and mutants defective in vesicle fusion (*sec17-1, sec18-1*) (Table III). Interactions with docking or fusion mutants were not observed for *sec12, sec13, sec16 or sec23* mutants (Kaiser and Schekman, 1990), suggesting that Sec24p performs a unique role among vesicle formation genes. We propose that Sec24p, like Iss1p, participates in docking of ER-derived vesicles with the Golgi apparatus.

DISCUSSION

In this paper, we describe a Sec24p homolog, Iss1p, that is similar to Sec24p in many respects: First, Iss1p has a high degree of sequence identity with Sec24p. Second, Iss1p physically interacts with two Sec24p binding proteins, Sec23p and Sec16p, and these interactions likely occur through similar domains of Iss1p. Finally, Iss1p has functional overlap with Sec24p, as evidenced by the observation that mutants in both genes show synthetic lethal interactions with vesicle docking mutants, and by the ability of *ISS1* to suppress a *sec24* mutant.

Given these similarities between Iss1p and Sec24p, it was surprising that Iss1p did not share the genetic interactions of a *sec24* mutant with mutants defective in vesicle formation, suggesting that the function of Iss1p is not required during vesicle formation. The functional differences between Iss1p and Sec24p may be due to subtle differences in the strength of the interactions with Sec23p and Sec16p, or they may reflect as yet unidentified properties, such as binding to an additional protein. However, since *ISS1* overexpression suppresses a *sec24* mutant that is at least partly defective in vesicle formation, it is likely that Iss1p can function during vesicle formation upon overexpression. It will be interesting to determine whether Iss1p can functionally substitute for Sec24p in the in vitro vesicle formation assay, and whether Iss1p is incorporated into vesicles like Sec24p.

Perhaps the most striking finding in this paper is that Iss1p and its homolog, the COPII coat component Sec24p, are important for docking of ER-derived vesicles with the Golgi apparatus. Evidence for this function comes from two observations. First, loss of function mutants in *iss1* and *sec24* exacerbate the growth defect of mutants defective in vesicle docking. Second, overexpression of *ISS1* suppresses the vesicle docking mutant *sec22*. The

genetic interactions of *sec24* mutants with vesicle docking mutants were particularly surprising, since none of the other mutants in COPII components shows interactions with vesicle docking mutants (Kaiser and Schekman, 1990).

Three mechanisms can be imagined by which Sec24p and Iss1p could affect vesicle docking. First, Sec24p and Iss1p may be required for loading of docking factors, such as Sec22p, Bos1p and, possibly, Bet1p, into vesicles during vesicle formation. Components of clathrin and coatomer coated vesicles have been identified that load cargo proteins into vesicles by binding to their cytosolic domains (Ohno et al., 1995; Cosson and Letourneur, 1994). It is conceivable that Sec24p and Iss1p similarly direct incorporation of docking factors into COPII coated vesicles by binding to their cytosolic domains. So far, attempts to coprecipitate Sec22p with either Sec24p or Iss1p have been unsuccessful (R. E. Gimeno, unpublished). A second possibility is that Sec24p and Iss1p participate in the formation of retrograde transport vesicles that recycle integral membrane docking factors to the ER. If formation of these vesicles is blocked, Sec22p and other docking factors will be depleted from the ER, ultimately causing a defect in vesicle docking. Interestingly, mutants in coatomer components that block recycling (sec21, sec26, sec27) have multiple genetic interactions with docking mutants similar to *iss1* and *sec24* mutants (Newman and Ferro-Novick, 1987; Duden et al., 1994). If Iss1p and Sec24p indeed participate in retrograde transport, it would be expected that mutants in these proteins cause missorting of recycled cargo proteins. So far, however, we have been unable to detect a sorting defect in *iss1* mutants. In particular, deletion of *iss1* does not cause missorting of Kar2p, an ER protein that undergoes recycling, and does not affect the recycling of a KKXX-containing fusion protein (our unpublished data). Finally, a role for Sec24p and Iss1p in vesicle docking could reflect a requirement for these proteins in disassembly of the COPII coat,

possibly stimulating GTP hydrolysis on Sar1p. This possibility is particularly intriguing, since both Sec24p and Iss1p bind to Sec23p, a protein that activates GTP hydrolysis by Sar1p. While Sec24p by itself does not affect the GAP activity of Sec23p, proteins that bind to Sec24p, such as Sec16p, may modulate its ability to increase the GAP activity of Sec23p. More detailed biochemical studies will be required to determine the effects of Sec24p and Iss1p on cargo protein loading and on the assembly and dissassembly of the COPII coat.

One other COPII coat component, Sar1p, has previously been implicated in vesicle docking. GTP hydrolysis on Sar1p is required for disassembly of the COPII coat after vesicle formation in vitro (Barlowe et al., 1994), and is also necessary for docking and fusion of ER-derived vesicles with the Golgi apparatus (Oka and Nakano, 1994). It might be expected that mutants in Sar1p that are locked predominantly in the GTP-bound form have genetic interactions similar to *sec24* mutants. Although mutants in other COPII coat components, such as *sec12*, *sec13*, *sec16*, *sec23* and *sec31*, do not show genetic interactions indicative of a role in vesicle docking (Kaiser and Schekman, 1990; Frand and Kaiser, unpublished), we cannot exclude the possibility that some of these proteins function during vesicle docking; isolation of new alleles may be required to reveal this additional function for COPII proteins in vivo.

The role of the COPII coat components in processes other than vesicle formation is still only poorly understood. The isolation of a homolog of a COPII coat component that is required for vesicle docking, but not vesicle formation, may provide an important tool to analyze the role of coat components in vesicle docking, and may shed light on the role of Sec24p in vesicle docking and fusion.

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Figure 1. (A) Comparison of the protein sequences of Iss1p and Sec24p. Lines indicate identical amino acids, dots indicate similar amino acids. Overall amino acid identity is 62%. The putative Zn-binding motif is printed in bold. Amino acid positions are indicated by the numbers to the right of each protein.

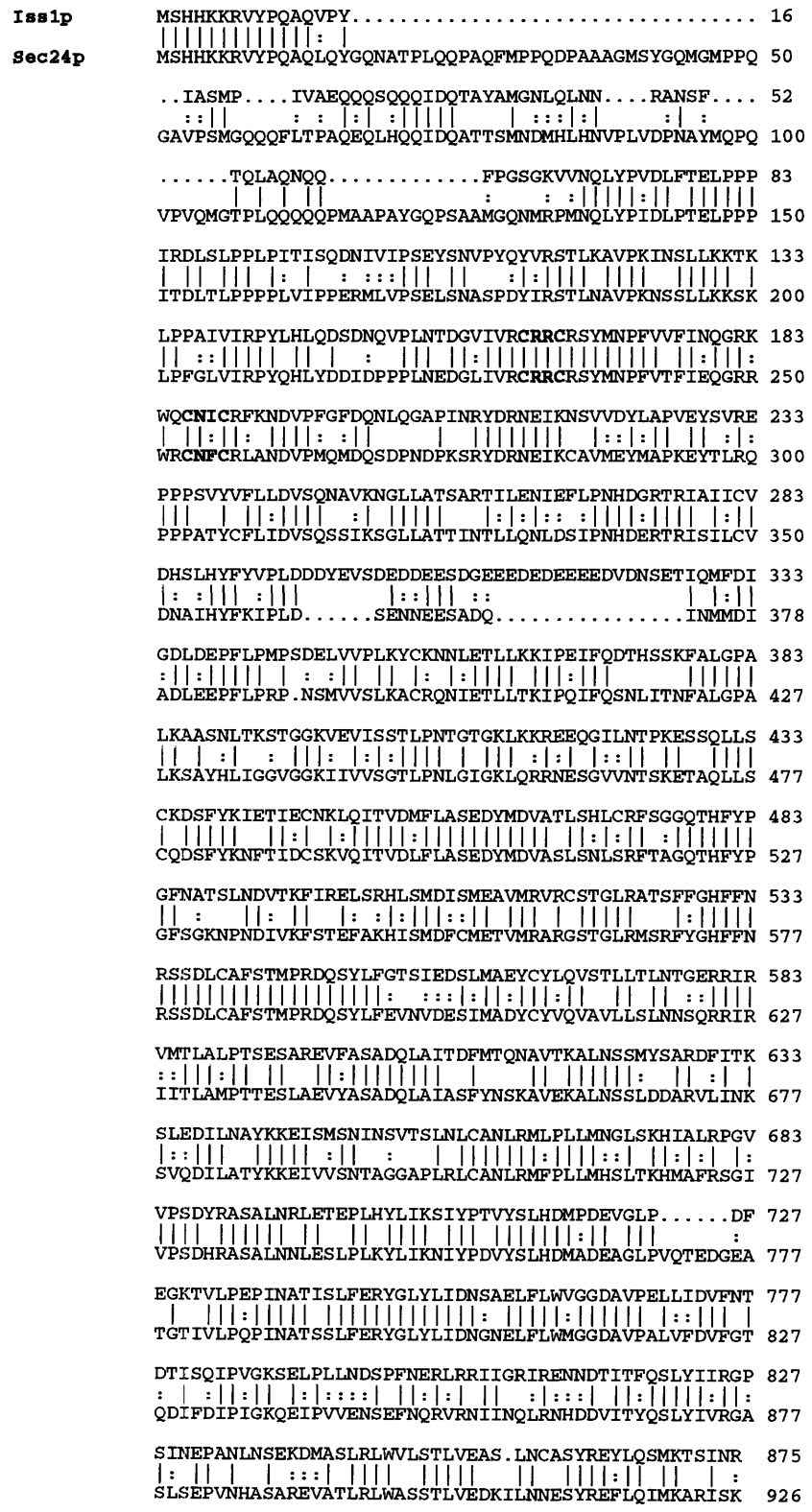

Iss1p
Sec24p

Figure 1. (B) Comparison of the putative Zn-binding motif of Sec24p, Iss1p, Lst1p and Sec23p. Amino acids that are identical in three of the four sequences are printed in bold; conserved cysteines are underlined. Amino acid positions are indicated by the numbers to the left of each sequence. The overall percent identity for the sequences shown is indicated to the right.

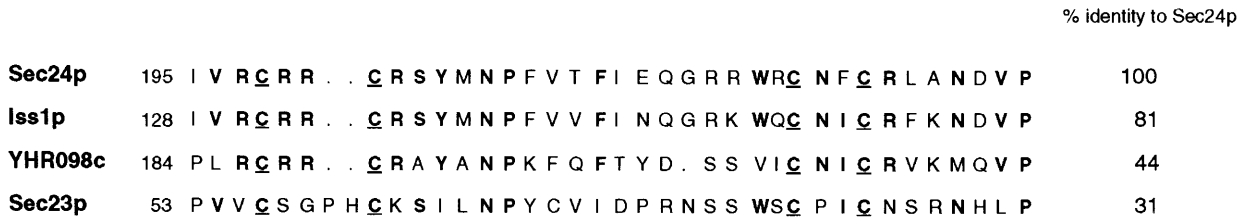
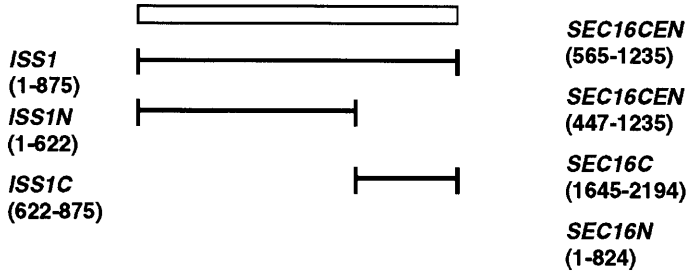

% identity to Sec24p
Sec24p 195 I V R C R R . . C R S Y M N P F V T F I E Q G R R W R C N F C R L A N D V P 100
Iss1p 128 I V R C R R . . C R S Y M N P F V V F I N Q G R K W Q C N I C R F K N D V P 81
YHR098c 184 P L R C R R . . C R A Y A N P K F Q F T Y D . S S V I C N I C R V K M Q V P 44
Sec23p 53 P V V C S G P H C K S I L N P Y C V I D P R N S S W S C P I C N S R N H L P 31

Figure 2. Two-hybrid interactions between Sec16p, Sec23p and Iss1p. The extent of each of the gene fragments of *ISS1* 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 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)		
ISS1 **(1-875)**	**221**	**5065**	< 20
ISS1N **(1-622)**	< 20	**127**	< 20
ISS1C **(622-875)**	**1566**	< 20	< 20
SEC23 **(1-768)**	< 20	66	33



B

LexA-DNA Binding Domain Fused to	Activation Domain fused to: *ISS1C* (622-875)	*SEC23* (1-768)	no fusion
	β-galactosidase activity (U)		
SEC16N **(1-824)**	26	< 20	< 20
SEC16CEN **(447-1235)**	**815**	42	< 20
SEC16C **(1645-2194)**	< 20	**3651**	< 20

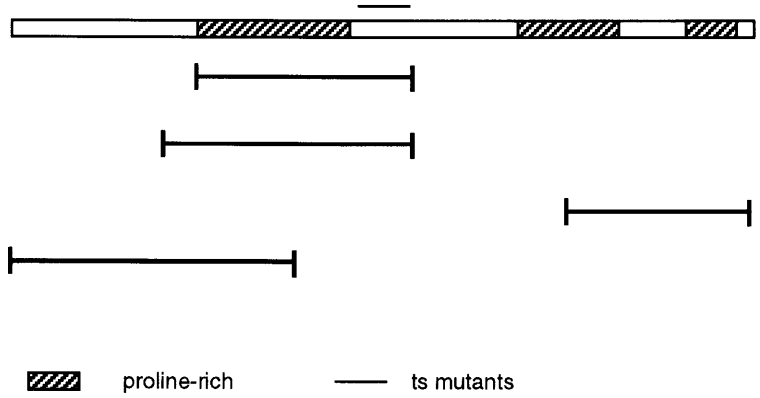


Figure 3. Affinity isolation of complexes between Iss1p, Sec16p and Sec23p from yeast extracts. Iss1p fused to GST (lanes 1 and 2) or GST only (lanes 3 and 4) were coexpressed with either the central domain of Sec16p fused to invertase (pRH317, upper panel) or Sec23p (pPE123, lower panel). GST-fusion 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 and 3: 100,000 x *g* supernatant from 0.044 OD_{600} U of cells. Lanes 2 and 4: material bound to beads from 0.73 OD_{600} U of cells.

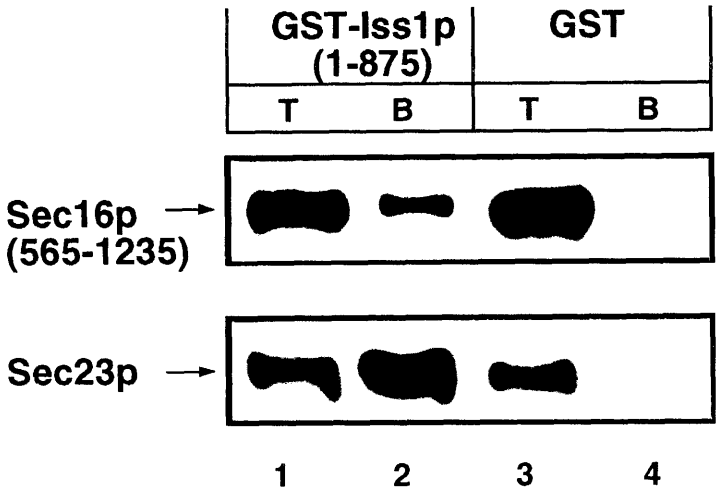

GST-Iss1p
(1-875)
GST
T
B
T
B
Sec16p
(565-1235)
Sec23p
1
2
3
4

Figure 4. ISS1 overexpression suppresses the temperature-sensitivity of *sec22-3*. A *sec22-3* strain (CKY70) containing vector (pRS306-2μ) or *ISS1* on a 2μ plasmid (pRH310), and a wild-type strain (CKY8) containing vector (pRS306-2μ) were streaked for single colonies on selective medium and incubated at 30°C for 40 h.

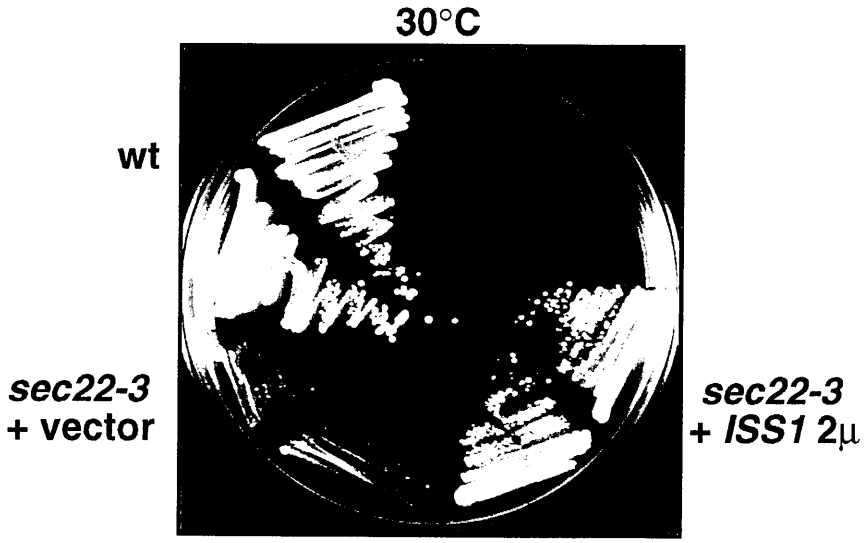
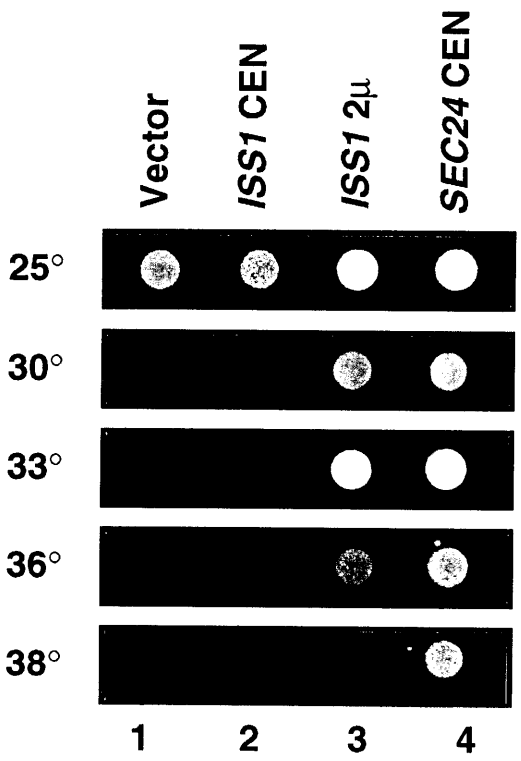

30°C
wt
sec22-3
+ vector
sec22-3
+ ISS1 2μ

Figure 5. *ISS1* overexpression suppresses the temperature-sensitive growth and secretion defect of *sec24-1*. (A) A *sec24-1* strain (CKY496) containing vector (pRS306-2μ, lane 1), *ISS1* on a CEN or 2μ plasmid (pRH200 and pRH310, lanes 2 and 3) or *SEC24* on a CEN plasmid (pAF70, lane 4). Cells were spotted on selective medium and incubated at the indicated temperatures for 40 h. (B) A *sec24-1* strain (CKY496) containing vector (pRS306-2μ, lanes 1, 4, 7), *ISS1* on a 2μ plasmid (pRH310, lanes 2, 5, 8) or *SEC24* on a CEN plasmid (pAF70, lanes 3, 6, 9) was grown in selective medium at 25°C and shifted to the indicated temperature for 1 h. Extracts of 0.2 OD_{600} U of cells were resolved by SDS-PAGE on an 8% gel and CPY was detected by Western blotting.

A


Vector
ISS1 CEN
ISS1 2μ
SEC24 CEN
25°
30°
33°
36°
38°
1
2
3
4

B

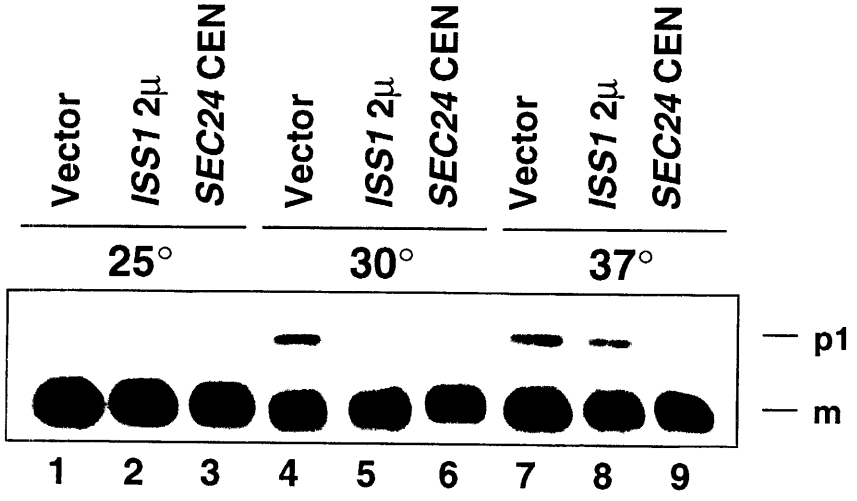

Vector
ISS1 2μ
SEC24 CEN
Vector
ISS1 2μ
SEC24 CEN
Vector
ISS1 2μ
SEC24 CEN
25°
30°
37°
p1
m
1
2
3
4
5
6
7
8
9

Table I. *S. cerevisiae* Strains

Strain	Genotype	Source or Reference
CKY8	*MATα ura3-52 leu2-3*	Kaiser Lab Collection
CKY19	*MATa/MATα ura3-52/ura3-52 leu2-Δ1/leu2-Δ1 his3-Δ200/his3-Δ200 lys2-801/lys2/801 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63*	Kaiser Lab Collection
CKY473	*MATα ura3-52 leu2-3,112* Gal$^+$	Kaiser Lab Collection
CKY496	*MATα sec24-1 ura3-52 leu2-3,112*	This study
CKY498	*MATa/MATα iss1-Δ1::TRP1/ISS1 ura3-52/ura3-52 leu2-Δ1/leu2-Δ1 his3-Δ200/his3-Δ200 lys2-801/lys2/801 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63*	This study
CKY499	*MATα iss1-Δ1::TRP1 ura3-52 leu2-Δ1 his3-Δ200 lys2-801 ade2-101 trp1-Δ63*	This study
CKY500	*MATa iss1-Δ1::TRP1 ura3-52 leu2-Δ1 his3-Δ200 lys2-801 ade2-101 trp1-Δ63*	This study
CKY39	*MATα sec12-4 ura3-52 his4-619*	Kaiser Lab Collection
CKY40	*MATa sec12-4 ura3-52*	Kaiser Lab Collection
CKY46	*MATa sec13-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY51	*MATa sec16-2 ura3-52*	Kaiser Lab Collection
CKY55	*MATa sec17-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY58	*MATα sec18-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY59	*MATa sec18-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY70	*MATα sec22-3 ura3-52 his4-619*	Kaiser Lab Collection
CKY71	*MATa sec22-3 ura3-52*	Kaiser Lab Collection
CKY72	*MATα sec22-1 ura3*	Kaiser Lab Collection
CKY78	*MATa sec23-1 ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY85	*MATa bet1-1 ura3-52*	Kaiser Lab Collection

Table II. 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
pRS306-2μ	2μ vector marked with *URA3*	Gimeno et al., 1995
pCT3	centromere vector marked with *URA3*	Thompson et al., 1993
pRH200	*ISS1* in pCT3	This study
pRH310	*ISS1* in pRS306-2μ	This study
pAF70	*SEC24* in pCT3	Gimeno et al., in press
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
pRH266	*ISS1* (1-875) in pEG202	This study
pRH319	*ISS1* (1-622) in pEG202	This study
pRH249	*ISS1* (622-875) in pEG202	This study
pDS79	*SEC23* in pGILDA	Gimeno et al., in press
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	Gimeno et al., in press
pPE81	*SEC23* in pJG4-5	Espenshade et al., 1995
pPE167	*SEC16* (565-1235) in pGAD-GH	Gimeno et al., in press
pRH343	*SEC24* (622-875) in pJG4-5	This study
pRD56	*GAL1* promoted *GST* in pRS316	Espenshade et al., 1995
pRH303	*GAL1* promoted *GST-ISS1* (1-875) in pRS316	This study
pRH317	*GAL1* promoted *SEC16* (565-1235)-*SUC2* in pRS315	Gimeno et al., in press
pPE123	*GAL1* promoted *SEC23* in pRS315	Gimeno et al., in press

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

Table III. Genetic Interactions of *iss1* and *sec24* Mutants

Genotype	Incubation Temperature		
	28°	30°	33°
sec24-1	++	–	–
sec24-1 iss1-Δ1::TRP1	++	–	–
A. Vesicle docking:			
sec22-3	++	±	–
sec22-3 iss1-Δ1::TRP1	double mutants not viable at 25°C		
sec22-3 sec24-1	double mutants not viable at 25°C		
bet1-1	+++	+++	+++
bet1-1 iss1-Δ1::TRP1	+++	–	–
bet1-1 sec24-1	double mutants not viable at 25°C		
B. Vesicle fusion:			
sec17-1	+++	++	±
sec17-1 iss1-Δ1::TRP1	+++	++	±
sec17-1 sec24-1	–	–	–
sec18-1	+++	–	–
sec18-1 iss1-Δ1::TRP1	+++	–	–
sec18-1 sec24-1	double mutants not viable at 25°C		
C. Vesicle formation:			
sec12-4	+++	–	–
sec12-4 iss1-Δ1::TRP1	+++	–	–
sec12-4 sec24-1	double mutants not viable at 25°C		
sec13-1	+++	+	–
sec13-1 iss1-Δ1::TRP1	+++	+	–
sec13-1 sec24-1	double mutants not viable at 25°C		
sec16-2	++	–	–
sec16-2 iss1-Δ1::TRP1	++	–	–
sec16-2 sec24-1	double mutants not viable at 25°C		
sec23-1	+++	–	–
sec23-1 iss1-Δ1::TRP1	+++	–	–
sec23-1 sec24-1	double mutants not viable at 25°C		

Growth of single colonies on YPD after 24 to 48h. +++, growth comparable to wild-type; –, no growth.

Chapter 5

Prospectus

This thesis focuses on the function of the COPII coat component Sec16p. Chapter 2 demonstrates that Sec16p interacts independently with two soluble COPII coat components, Sec23p and Sec24p. The ability of Sec16p to bind to soluble COPII coat components suggests that Sec16p may act as a scaffold for the assembly of cytosolic coat components. Chapter 3 describes Sed4p, an integral ER membrane component that has properties expected of a regulator of COPII coat assembly. Interestingly, Sed4p, like Sec23p, interacts with the C-terminal domain of Sec16p. Since Sed4p, Sec23p and Sec16p have strong genetic and/or biochemical interactions with Sar1p, a protein that is thought to initiate COPII vesicle formation, we proposed that initiation of vesicle formation occurs at the C-terminal domain of Sec16p (Chapter 2). Our model for COPII vesicle formation suggests that Sar1p primes Sec16p for binding of Sec23p; Sec23p binding then allows assembly of Sec24p, which in turn recruits Sec13p and Sec31p.

This model for COPII coat assembly can be tested using tools developed in the course of this thesis. In particular, it would be expected that binding of Sec23p/Sec24p and Sec13p/Sec31p to Sec16p is regulated by Sar1p. Using pure Sec23p and Sec16p as described in Chapter 2, bacterially expressed Sar1p (Barlowe et al., 1993) and bacterially expressed Sed4p N-terminus (R. E. Gimeno, unpublished), the effect of Sar1p on binding of Sed4p and Sec23p to Sec16p can now be examined in vitro. Preliminary experiments suggest that Sed4p and Sec23p bind to a similar site on Sec16p, as binding of Sed4p and Sec23p is mutually exclusive (P. Espenshade, personal communication). This finding is consistent with our proposal that Sed4p binds to Sec16p at the ER membrane prior to or during vesicle formation, while binding of Sec23p to Sec16p represents the first step in assembly of the COPII coat. It will be interesting to examine whether Sar1p can modulate the ratio of Sec23p to Sed4p

bound to Sec16p. In chapter 3 we suggest that Sec12p and Sed4p perform distinct functions. Sec12p does not associate with the C-terminal domain of Sec16p in coprecipitation experiments, and genetic tests show that Sec12p and Sed4p are functionally distinct. Using bacterially expressed Sec12p N-terminus (P. Espenshade, unpublished), it will now be possible to examine whether Sec12p is indeed unable to bind to the C-terminus of Sec16p in vitro. It will also be important to test whether Sec12p affects binding of Sec23p to Sec16p.

The two-hybrid system has proven to be useful for identifying proteins that bind to Sec16p. Conventional biochemical approaches to this problem were difficult since Sec16p is a large, easily degraded molecule that is solubilized only by treatment with agents that disrupt protein-protein interactions (0.5 M NaCl or pH 11). Using a two-hybrid screen similar to the one described in chapter 4, D. Shaywitz recently identified a third COPII coat component, Sec31p, as a Sec16p binding molecule (D. Shaywitz, unpublished). Interestingly, Sec31p binds to the central domain of Sec16p at a site close to the Sec24p binding site and interacts with Sec24p in both the two-hybrid assay and by coimmunoprecipitation (D. Shaywitz and R. E. Gimeno, unpublished data). Thus, Sec24p could recruit the Sec13p/Sec31p complex to the membrane both by binding directly to this complex and by facilitating its binding to Sec16p. Once purified Sec31p is available, it will be possible to test whether Sar1p or Sec23p facilitate the interaction between Sec24p and Sec31p and whether Sec24p facilitates the binding of Sec31p to Sec16p.

The COPII coat in vivo is likely not a stable complex, but goes through rounds of assembly and disassembly on the membrane. By identifying Sec16p as a scaffold on which this assembly could occur, and by developing assays in solution that allow the analysis of protein-protein interactions on Sec16p, it may

now be possible to reconstitute the subunit interaction steps that occur during COPII coat assembly in vitro.

Chapter 4 describes a new Sec16p binding protein, the Sec24p homolog Iss1p. Surprisingly, both Sec24p and Iss1p appear to function in docking of COPII coated vesicles. As described in the discussion of Chapter 4, this function of Sec24p and Iss1p could reflect a role for Sec24p/Iss1p in loading of docking components into the vesicle or a requirement for these proteins during steps preceding vesicle docking, such as uncoating. Both possibilities represent a novel function for COPII coat components and can be easily explored further. In particular, the importance of Iss1p in loading docking factors could be examined in vitro by determining the ability of membranes and cytosol from an *iss1* deletion strain to incorporate the docking factor Sec22p or a soluble cargo protein, such as radiolabeled α-factor, into vesicles. Furthermore, since the stability of the COPII coat has been tied to GTP-hydrolysis in Sar1p (Oka and Nakano, 1994), it would be interesting to examine the effects of Sec24p and Iss1p on the GAP activity of Sec23p in the presence or absence of Sec16p.

An important question in COPII coat formation is how cargo proteins are incorporated into vesicles. Appendix I describes a protein, Iss13p/Hkr1p, that was identified based on its interaction with the C-terminal domain of Sec16p in the two-hybrid assay. Iss13p appears to be an integral membrane cargo protein; it is tempting to speculate that the association between Sec16p and Iss13p plays a role in transport of Iss13p from the ER to the Golgi apparatus. It will be important to verify the interaction of Iss13p/Hkr1p with the C-terminal domain of Sec16p in vitro. If the cytosolic domain of Iss13p indeed binds to Sec16p, the effects of Iss13p on the protein-protein interactions described above can then be examined.

The finding that a putative cargo protein, Iss13p, as well as several other membrane proteins (Iss11p, Iss12p, Iss14p, Iss15p, Iss18p) interact with Sec16p in the two-hybrid assay suggests that Sec16p may have a general role in loading cargo proteins into COPII coated vesicles. Sec16p could bind directly to the cytosolic domains of integral membrane cargo proteins; soluble cargo proteins could be loaded into vesicles by binding to Sec16p of an integral membrane sorting receptor that binds to soluble cargo proteins. It will be interesting to examine whether Sec16p binds to the cytosolic domain of other known cargo proteins, such as Sec22p and Emp24p/Bst2p.

It is worth noting that Iss11p - Iss18p interact with both the C-terminal domain of Sec16p and with Sec23p in the two-hybrid assay, suggesting that cargo loading into COPII vesicles may involve a trimeric Sec23p/Sec16p/cargo protein complex. Interestingly, initiation of vesicle formation may also occur at the C-terminus of Sec16p and involves Sec23p (see above). Thus, cargo protein loading may be tightly coupled to initiation of COPII vesicle formation by facilitating the recruitment of cytosolic COPII coat components to Sec16p.

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Appendix I

Identification of Proteins that Interact with Sec16p Using the Two-Hybrid System

PREFACE

This appendix summarizes the results of two-hybrid screens for proteins that interact with the central or the C-terminal domain of Sec16p.

MATERIALS AND METHODS

Plasmids Constructions. Plasmids are summarized in Table I. To make pRH108, a fragment encoding amino acids 1-347 of *SED4* was amplified by PCR as described (Gimeno et al., 1995), and ligated into *EcoR*I-*Xho*I cut pEG202. pRH157 is the *BamH*I-*Sac*I fragment of a *GAL* promoted *SEC13* construct (Shaywitz et al., 1995) ligated into *BamH*I-*Xho*I cut pEG202X, a derivative of pEG202, in which the reading frame of the polylinker is changed. pRH221-229 are library plasmids recovered from the screen.

Two-Hybrid Screen for Proteins that Interact with Sec16p. Two-hybrid screens were performed as described (Chapter 4; Golemis et al., 1994). Plasmids containing either the central (amino acids 447-1235; pPE62) or the C-terminal (amino acids 1645-2194; pPE58) domain of Sec16p fused to the *lexA* DNA binding domain were used as a bait, and a *S. cerevisiae* genomic library fused to an acidic activation domain was used as a fish (Watt et al., 1995). Briefly, ~2 x 10^5 transformants were screened for Leu$^+$ β-Gal$^+$ colonies; plasmids were recovered from positive colonies and analyzed by restriction digestion and DNA sequencing.

Two-Hybrid Test for Protein-Protein Interactions. Protein-protein interactions were tested in the two-hybrid system using either a transformation (Golemis et al., 1994) or a mating assay (Finley and Brent, 1994). In the transformation assay, fish and bait constructs were cotransformed into EGY48 carrying the

indicator plasmid pSH18-34 (Golemis et al., 1994), and *lacZ* expression was tested by patching 4 to 8 transformants on SC medium (pH 7.0) lacking the appropriate amino acids and supplemented with 2% galactose and 40 mg/l X-gal. In the mating assay, fish constructs were transformed into EGY48 and bait constructs and the indicator plasmid pSH18-34 were transformed into RFY206 (Finley and Brent, 1994). Bait and fish constructs were then brought together by mating, and *lacZ* expression was tested by patching two diploids derived from two independent transformants on X-gal medium as above.

RESULTS AND DISCUSSION

Iss1p and Iss2p Interact with the Central Domain of Sec16p.

We have previously shown that the central domain of Sec16p (amino acids 447-1235) interacts with the COPII coat component Sec24p (see chapter 2). To identify additional proteins that bind to this domain of Sec16p, we performed a two-hybrid screen using a library of *Saccharomyces cerevisiae* genomic DNA. Two proteins, Iss1p and Iss2p (Interacting with Sec sixteen 1 and 2), interacted strongly with the central domain of Sec16p. The properties of Iss1p and Iss2p are summarized in Table II, and Iss1p is described in detail in Chapter 4. Iss2p is a novel, hydrophilic protein that is closely related to an uncharacterized yeast protein, YGL107c (Fig. 1), but has no homology to any other proteins in the database.

We tested the protein-protein interactions of the Iss2p fragment recovered in the screen using the two-hybrid system (Fig. 2). As expected, Iss2p interacted strongly with the central domain of Sec16p. Interestingly, Iss2p also showed strong interactions with Iss1p and Sec24p, two proteins that bind to the central domain of Sec16p (Gimeno et al., in press, and chapter 4). Iss2p interacted with both the N-terminal and the C-terminal domain of either Sec24p or Iss1p,

suggesting that Sec24p and Iss1p have at least two distinct binding sites for Iss2p. Iss2p did not interact with several protein that have previously been shown to be active in the two-hybrid assay, including the C-terminal domain of Sec16p, the N-terminal domain of Sed4p, or full-length Sec23p or Sec13p (Fig. 2). The finding that Iss2p interacts with only a small number of bait proteins strongly argues that the observed interactions for Iss2p are specific.

The two-hybrid data suggest that Iss2p may form a ternary complex with the COPII coat components Sec16p and Sec24p. Thus, Iss2p is an excellent candidate for a novel COPII coat associated protein. Iss2p could be part of the COPII coat, or it could interact with COPII coat components either before coat formation or after coat disassembly. It will be important to confirm the observed two-hybrid interactions of Iss2p in vitro. Furthermore, it will be interesting to determine whether deletion of *iss2* - either by itself or in combination with its homolog, YGL107c - affects transport from the ER to the Golgi apparatus and, if so, which step during transport is affected, and whether Iss2p localizes to ER-derived vesicles.

Iss11p - Iss18p Interact with the C-terminal Domain of Sec16p. The C-terminal domain of Sec16p binds to the COPII coat component Sec23p and to the cytosolic domain of an integral ER membrane protein, Sed4p (Gimeno et al., 1995; Gimeno et al., in press). To identify additional proteins that bind to the C-terminal domain of Sec16p, we performed a two-hybrid screen using the library described above. Eight proteins, Iss11p-18p, were identified. The properties of these proteins are summarized in Table III and Fig. 3.

Two of the eight proteins, Iss16p and Iss17p, are predicted to be soluble proteins. Both Iss16p and Iss17p are novel proteins with no homology to other proteins in the database. Iss17p was previously isolated as a high copy

suppressor of temperature-sensitive mutations in the coatomer component *arf1* (*SFS2*, Kahn, R. A., personal communication; Kahn et al., 1995), and is not essential for viability (C. A. Carter, personal communication). The genetic interaction of *ISS17* with *ARF1* suggests that Iss17p is indeed involved in the secretory pathway, although its exact role remains unclear. As discussed below, Iss16p interacts with Sec13p in the two-hybrid assay (Fig. 4).

The remaining six proteins identified as interacting with the C-terminal domain of Sec16p are predicted to be integral membrane proteins (Fig. 3). We were surprised to recover several fusion proteins that contained one or more membrane-spanning domains, since a two-hybrid signal is generally thought to require soluble bait and fish proteins that have been transported into the nucleus. Possibly, fusion proteins, once inserted in the ER membrane, can be translocated to the inner membrane of the nuclear envelope and then interact with the soluble bait protein in the nucleus. Alternatively, the presence of the acidic activation domain may disrupt insertion of some fusion proteins into the membrane, resulting in a soluble fish protein.

One of the six integral membrane proteins, Iss13p, has been previously identified as Hkr1p, a protein that confers resistance to HM-1 killer toxin when overexpressed (Kasahara et al., 1994). Iss13p is predicted to encode a type I integral membrane protein; the C-terminal, cytosolic domain of Iss13p consists of 294 amino acids and contains a calcium-binding consensus sequence (EF hand motif; Kasahara et al., 1994). Given the location of Sec16p on the cytosolic face of the membrane, it is expected that Sec16p interacts with the C-terminal, cytosolic domain of Iss13p. While cells carrying a deletion of the entire *ISS13* reading frame are inviable (Kasahara et al., 1994), cells carrying an allele of *ISS13* that is truncated just before the transmembrane domain show reduced β-1,3,-glucan synthase activity, an abnormal cell wall composition, and

abnormalities in budding pattern (Yabe et al., 1996). This argues that the transmembrane and/or cytosolic domains of Iss13p, are required for proper cell function. Iss13p is localized to the plasma membrane (Yabe et al., 1996); thus, any interaction between Iss13p and Sec16p is likely to occur during transit of Iss13p through the ER.

Two integral membrane proteins, Iss14p and Iss18p, show significant homology to proteins in the database. Iss14p has strong similarity to P-type ATPases. Interestingly, Iss14p has a C-terminal ER retention sequence (KXKXX), suggesting that Iss14p may function in the ER. Iss18p has strong similarity to the yeast protein Sge1p, that confers resistance to crystal violet when overexpressed (Ehrenhofer-Murray et al., 1994). Since Iss18p also has weaker similarity to a number of bacterial antibiotic resistance proteins, Iss18p is likely to be a membrane transport protein.

We used the two-hybrid system to test whether the Iss11p-18p fragments recovered in the screen interacted with proteins other than the C-terminal domain of Sec16p (Fig. 4). Interestingly, all of the fragments interacted with Sec23p, but none interacted with the N-terminal domain of Sed4p (Fig. 4). Furthermore, none of the fragments interacted with the central or the N-terminal domain of Sec16p, and only one fragment, Iss16p, interacted with Sec13p (Fig. 4). Importantly, the N-terminal domain of Sed4p used in this assay is active since it can interact with a C-terminal fragment of Sec16p (data not shown). These data suggest that Iss11p-18p bind specifically to the C-terminal domain of Sec16p and to Sec23p.

Since Iss11p-18p interact with both the C-terminal domain of Sec16p and with Sec23p, but not with Sed4p, we propose that Iss11p-18p interact with Sec16p during or after vesicle formation. Iss16p and Iss17p could be components of the COPII coat that regulate vesicle formation or affect the function of the vesicle after vesicle formation is complete. An intriguing possibility

for membrane-spanning Iss proteins is that these proteins are cargo proteins that interact with Sec16p during cargo sorting into vesicles. This hypothesis is consistent with the localization of Iss13p at the plasma membrane, and the finding that a large number of diverse proteins can bind to the C-terminal domain of Sec16p. Sec16p, perhaps in conjunction with Sec23p, may function as a cargo receptor during vesicle formation at the ER. Clearly, more work is needed to understand the role of Sec16p and its interactions with Iss proteins.

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Figure 1. Comparison of the protein sequences of Iss2p and YGL107c. Solid lines indicate identical amino acids, dotted lines indicate similar amino acids. Overall amino acid identity is 47%. Amino acid positions are indicated by the numbers to the right of each protein. Sequence comparison was performed using the BestFit program (Genetics Computer Group).

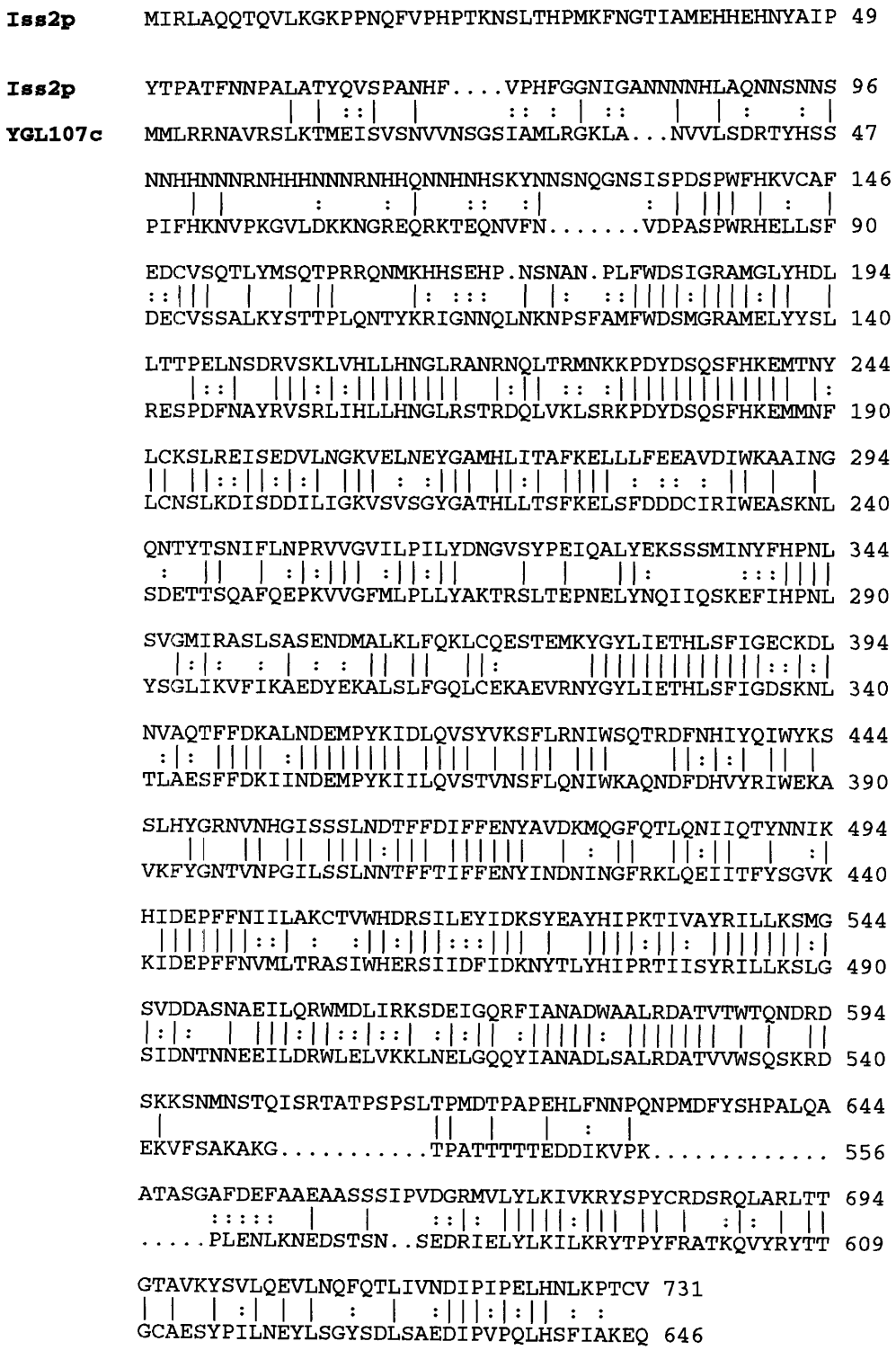
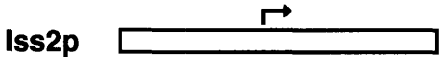

Iss2p MIRLAQQTQVLKGKPPNQFVPHPTKNSLTHPMKFNGTIAMEHHEHNYAIP 49
Iss2p YTPATFNNPALATYQVSPANHF....VPHFGGNIGANNNNHLAQNNSNNS 96
YGL107c MMLRRNAVRSLKTMEISVSNVVNSGSIAMLRGKLA...NVVLSDRTYHSS 47
NNHHNNNRNHHHNNNRNHHQNNHNHSKYNNSNQGNSISPDSPWFHKVCAF 146
PIFHKNVPKGVLDKKNGREQRKTEQNVFN.......VDPASPWRHELLSF 90
EDCVSQTLYMSQTPRRQNMKHHSEHP.NSNAN.PLFWDSIGRAMGLYHDL 194
DECVSSALKYSTTPLQNTYKRIGNNQLNKNPSFAMFWDSMGRAMELYYSL 140
LTTPELNSDRVSKLVHLLHNGLRANRNQLTRMNKKPDYDSQSFHKEMTNY 244
RESPDFNAYRVSRLIHLLHNGLRSTRDQLVKLSRKPDYDSQSFHKEMMNF 190
LCKSLREISEDVLNGKVELNEYGAMHLITAFKELLLFEEAVDIWKAAING 294
LCNSLKDISDDILIGKVSVSGYGATHLLTSFKELSFDDDCIRIWEASKNL 240
QNTYTSNIFLNPRVVGVILPILYDNGVSYPEIQALYEKSSSMINYFHPNL 344
SDETTSQAFQEPKVVGFMLPLLYAKTRSLTEPNELYNQIIQSKEFIHPNL 290
SVGMIRASLSASENDMALKLFQKLCQESTEMKYGYLIETHLSFIGECKDL 394
YSGLIKVFIKAEDYEKALSLFGQLCEKAEVRNYGYLIETHLSFIGDSKNL 340
NVAQTFFDKALNDEMPYKIDLQVSYVKSFLRNIWSQTRDFNHIYQIWYKS 444
TLAESFFDKIINDEMPYKIILQVSTVNSFLQNIWKAQNDFDHVYRIWEKA 390
SLHYGRNVNHGISSSLNDTFFDIFFENYAVDKMQGFQTLQNIIQTYNNIK 494
VKFYGNTVNPGILSSLNNTFFTIFFENYINDNINGFRKLQEIITFYSGVK 440
HIDEPFFNIILAKCTVWHDRSILEYIDKSYEAYHIPKTIVAYRILLKSMG 544
KIDEPFFNVMLTRASIWHERSIIDFIDKNYTLYHIPRTIISYRILLKSLG 490
SVDDASNAEILQRWMDLIRKSDEIGQRFIANADWAALRDATVTWTQNDRD 594
SIDNTNNEEILDRWLELVKKLNELGQQYIANADLSALRDATVVWSQSKRD 540
SKKSNMNSTQISRTATPSPSLTPMDTPAPEHLFNNPQNPMDFYSHPALQA 644
EKVFSAKAKG.....TPATTTTTEDDIKVPK..... 556
ATASGAFDEFAAEAASSSIPVDGRMVLYLKIVKRYSPYCRDSRQLARLTT 694
.....PLENLKNEDSTSN..SEDRIELYLKILKRYTPYFRATKQVYRYTT 609
GTAVKYSVLQEVLNQFQTLIVNDIPIPELHNLKPTCV 731
GCAESYPILNEYLSGYSDLSAEDIPVPQLHSFIAKEQ 646

Figure 2. Two-hybrid interactions of Iss2p. The amino acid numbers included in each construct are given in brackets. The portion of Iss2p used is indicated by the arrow on the diagram. Constructs marked with an asterisk were tested in the mating assay, unmarked constructs were tested in the transformation assay. 4-8 transformants (transformation assay) or 2 independent diploids (mating assay) were patched on medium containing X-gal and their color was evaluated after 24 to 48 h. **+++**, dark blue; —, white.

LexA-DNA Binding Domain Fused to	Activation Domain fused to *ISS2* (329-731)	Activation Domain fused to no fusion
	β-galactosidase activity (Blue color on X-GAL plates)	
SEC16CEN **(447-1235)**	+++	—
SEC16C **(1645-2194)**	—	—
SED4N **(1-347)**	—	—
SEC13* **(1-297)**	—	—
SEC23 **(1-768)**	—	—
SEC24 **(1-926)**	+++	—
SEC24N **(1-666)**	+++	—
SEC24C **(666-926)**	+++	—
ISS1 **(1-875)**	+++	—
ISS1N **(1-622)**	+	—
ISS1C **(622-875)**	+++	—



↱ Starting Point of Clone Identified in Screen

Figure 3. Schematic representation of Iss11p - Iss18p. The starting points of the clones identified in the screen are indicated by an arrow. Transmembrane domains are indicated by shaded boxes. Transmembrane domains were predicted using the TMpred program (Hofmann and Stoffel, 1993; http://ulrec3.unil.ch/software/TMPRED_form.html)

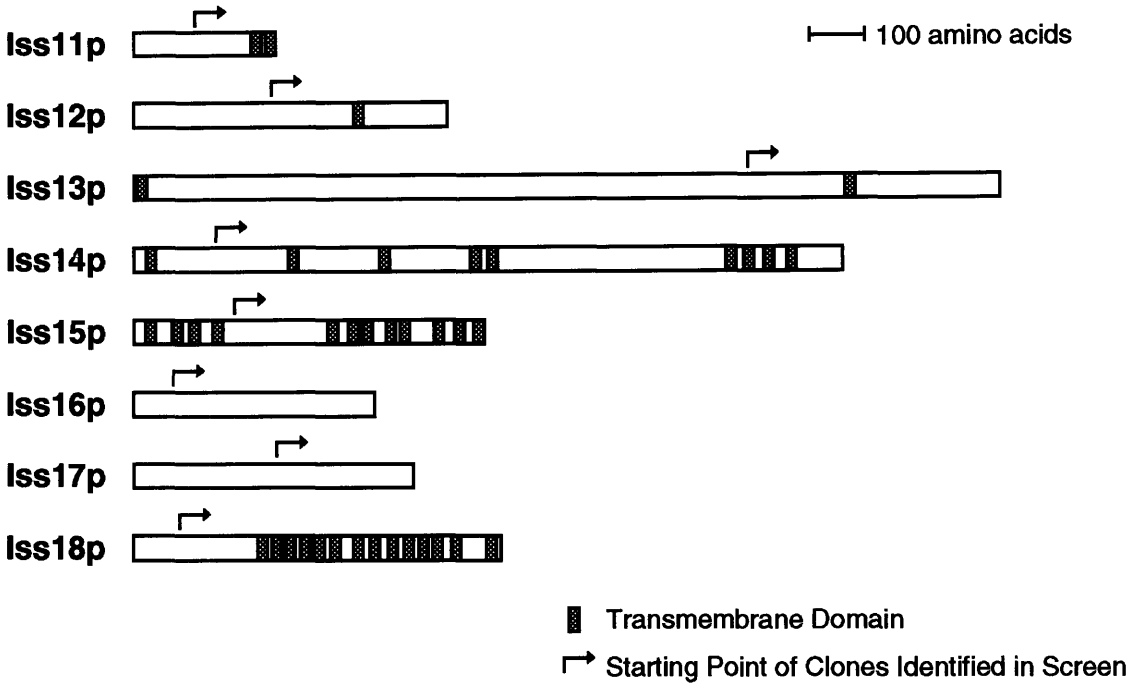

Iss11p
Iss12p
Iss13p
Iss14p
Iss15p
Iss16p
Iss17p
Iss18p
100 amino acids
Transmembrane Domain
Starting Point of Clones Identified in Screen

Figure 4. Two-hybrid interactions of Iss11p-Iss18p. The amino acid numbers included in each construct are given in brackets. Constructs marked with an asterisk were tested in the mating assay, unmarked constructs were tested in the transformation assay. 4-8 transformants (transformation assay) or 2 independent diploids (mating assay) were patched on medium containing X-gal and their color was evaluated after 24 to 48 h. **+++**, dark blue; **—**, white.

LexA-DNA Binding Domain Fused to	Activation Domain fused to								
	ISS11 **(127-292)**	***ISS12*** **(281-656)**	***ISS13*** **(1263-1802)**	***ISS14*** **(160-1472)**	***ISS15*** **(200-725)**	***ISS16*** **(85-500)**	***ISS17*** **(298-586)**	***ISS18*** **(94-768)**	**no fusion**
	β-galactosidase activity (Blue color on X-GAL plates)								
SEC16C **(1645-2194)**	+++	+++	+++	+++	+++	++	++	++	—
SEC23 **(1-768)**	+++	+++	+	+	+++	+	+++	+++	—
SEC13* **(1-297)**	—	—	—	—	—	++	—	—	—
SEC16CEN* **(447-1235)**	—	—	—	—	—	—	—	—	—
SEC16N* **(1-824)**	—	—	—	—	—	—	—	—	—
SED4N **(1-347)**	—	—	—	—	—	—	—	—	—

Table I. Plasmids

Plasmid	Description	Source or Reference
pEG202	*lexA* DNA binding domain in a 2μ vector marked with *HIS3*	Golemis et al., 1994
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*	Golemis et al., 1994
pSH18-34	*lacz* gene under control of 8 *lexA* DNA binding sites in a 2μ vector marked with *URA3*	Golemis et al., 1994
pRH229	*ISS2* (329-731) in pJG4-5	This study
pRH221	*ISS11* (127-292) in pJG4-5	This study
pRH222	*ISS12* (281-656) in pJG4-5	This study
pRH223	*ISS13* (1263-1802) in pJG4-5	This study
pRH224	*ISS14* (160-1472) in pJG4-5	This study
pRH225	*ISS15* (200-725) in pJG4-5	This study
pRH226	*ISS16* (85-500) in pJG4-5	This study
pRH227	*ISS17* (298-586) in pJG4-5	This study
pRH228	*ISS18* (94-768) in pJG4-5	This study
pRH108	*SED4* (1-347) in pEG202	This study
pRH157	*SEC13* in pEG202	This study
pDS79	*SEC23* in pGILDA	Gimeno et al., in press
pRH266	*ISS1* (1-875) in pEG202	Chapter 4
pRH319	*ISS1* (1-622) in pEG202	Chapter 4
pRH249	*ISS1* (622-875) in pEG202	Chapter 4
pRH286	*SEC24* (34-926) in pEG202	Gimeno et al., in press
pRH321	*SEC24* (1-666) in pEG202	Gimeno et al., in press
pRH347	*SEC24* (666-926) in pEG202	Gimeno et al., in press
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	Gimeno et al., in press

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

Table II. Proteins Identified in the Two-Hybrid Screen that Interact with the Central Domain of Sec16p

Name	Homolog/Function	Properties	Accession Number/ Reference
Iss1p	Sec24p	99 kD hydrophilic protein; binds to Sec23p	see Chapter 4
Iss2p	YGL107c	84 kD hydrophilic protein; interacts with Sec24p and Iss1p	Z36107

Table III. Proteins Identified in the Two-Hybrid Screen that Interact with the C-terminal Domain of Sec16p

Name	Homolog/Function	Properties	Accession Number/ Reference
Iss11p		33 kD membrane protein	X92441
Iss12p		74 kD membrane protein	Z46659
Iss13p/ Hkr1p	Msb2p*	189 kD type I plasma membrane protein; EF-hand motif in cytosolic domain; transmembrane and/or cytosolic domain essential for normal cell wall composition; extracellular domain essential for viability	S69101; Kasahara et al., 1994; Yabe et al., 1996
Iss14p	P-type ATPases	167 kD polytopic membrane protein; C-terminal ER retention motif (KXKXX)	Z75199
Iss15p		81 kD polytopic membrane protein	Z49606
Iss16p		57 kD hydrophilic protein; two-hybrid interaction with Sec13p	U32517
Iss17p/ Sfs2p		65 kD hydrophilic protein; not essential for viability; multicopy suppressor of arf1 mutants	X59720; Kahn et al., 1995
Iss18p	drug resistance proteins	86 kD polytopic membrane protein	Z48758

* Msb2p and Hkr1p are of similar size and topology, but amino acid similarity is restricted to two 60-70 aa regions immediately preceding the transmembrane domain.

Appendix II

PREFACE

Appendix II represents primarily Peter Espenshade's work. My main contribution is the development of the two-hybrid system to assay interactions of Sec16p with Sec23p (Table III).

Appendix II has been published in its entirety in the Journal of Cell Biology as: Espenshade, P., Gimeno, R. E., Holzmacher, E., Teung, P., and C. A. Kaiser. (1995). Yeast *SEC16* gene encode a multidomain vesicle coat protein that interacts with Sec23p. *J. Cell Biol.* 131:311-324.

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 N-terminus of the protein, defining a second functional domain. A separate function for the C-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.