Towards an Understanding of the Role of COPII Subunits in Vesicle Coat Assembly

by

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Abstract:

In eukaryotic cells, proteins traversing the secretory pathway are transported from the endoplasmic reticulum (ER) to the Golgi apparatus by membrane-bounded vesicles formed by the recruitment and assembly of cytosolic coat components upon the ER membrane. Three cytosolic factors, collectively termed COPII, have been shown to be required for vesicle formation in the yeast *S. cerevisiae*: the Sec23p/Sec24p protein complex, the Sec13p/Sec31p protein complex, and the small GTP-binding protein Sar1p. Recent evidence suggests that the membrane-associated protein Sec16p also participates in the formation of the vesicle coat, and may be considered an additional component of the COPII complex.

To better understand the role of COPII subunits, we have adopted two different approaches:

1. Identification of yeast proteins that are associated with COPII subunits. We have found that Sec31p binds not only Sec13p, but also Sec16p, Sec23p, and Sec24p. A different region of Sec31p appears to mediate each of these interactions. In addition, we have shown that the binding of Sec31p to Sec16p is necessary for ER to Golgi transport. We propose that Sec16p organizes the assembly of a coat which is stabilized both by the interaction of Sec31p with Sec23p and Sec24p, as well as by the interaction of all three of these components with Sec16p.

2. Identification and characterization of COPII homologs in mammalian cells. We have shown that although the mammalian homolog of *SEC13*, *Sec13R*, does not complement a *sec13-1* mutant, chimeric constructs representing the N-terminal half of either Sec13p or Sec13Rp, and the C-terminal half of the other protein, are able to complement the *sec13-1* secretion defect. We have also demonstrated that in mammalian cells, Sec13Rp is localized to the cisternae and vesicles in the region of the transitional ER. In addition, we have shown that yeast Sec13p and human Sec13Rp both interact with the same region of yeast Sec31p. Collectively, these results suggest that the function of Sec13p -- and of the COPII coat as a whole -- has been evolutionarily conserved.

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Title: Associate Professor of Biology

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Chapter I

COPII coat proteins: mediators of vesicle formation and cargo selection at the endoplasmic reticulum

PREFACE

Portions of this chapter are in press 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

A defining characteristic of eukaryotic cells is the presence of organelles -- distinct, membrane-enclosed compartments evolved to host a specific set of biochemical reactions. The ability to create enclosed intracellular spaces presumably affords the eukaryotic cell greater opportunity to utilize processes that would be difficult to carry out in a less specialized biochemical environment. Thus, while proteins synthesized by bacteria are compelled to mature under essentially identical conditions -- the chemical environment of the bacterial cytoplasm -- proteins synthesized by eukaryotic cells are afforded two potential pathways: they can either fold in the cytoplasm or enter the specialized organelles comprising the secretory pathway. These organelles -- and the mechanism of transport between them -- will be the focus of this discussion.

Almost every eukaryotic protein destined either for release into the extracellular space or for residence in the plasma membrane or the lysosome (vacuole in yeast) travels through a defined series of organelles, starting with the endoplasmic reticulum (ER), and continuing through the *cis-*, *medial-*, and *trans*-Golgi. Travel from one compartment to another is mediated by membrane-bounded vesicles which bud from the donor compartment and later fuse with the target organelle (Palade, 1975). In the most fully understood examples of protein transport between organelles, vesicle budding is accompanied by the assembly upon the membrane of a coat that is derived from soluble cytosolic proteins. The coat is thought to contribute to both the formation of the vesicle and the selection of its cargo; this dual role is particularly important because it implies that coat proteins are information-

rich. Consequently, the study of coat proteins is likely to yield important insights into both the biomechanics of vesicle formation as well as the regulation of cargo transport.

Three different types of coated vesicles have been well-characterized in yeast and mammalian cells. The first class of coated vesicles to be discovered has a cytosolic coat composed of an outer lattice of clathrin and four additional proteins that constitute the adaptor protein (AP) complex (Pearse and Robinson, 1990). Clathrin-coated vesicles in mammalian cells are responsible for transport steps directed to the lysosome either from the plasma membrane or from the trans-Golgi compartment. A second type of vesicle coat, known as COPI, was discovered by cell-free reconstitution of transport between Golgi compartments. These vesicles are coated with a complex of seven proteins known as coatomer (Rothman 1994). The involvement of COPI vesicles in transport through the Golgi has yet to be established definitively in vivo. Experiments in yeast have shown that COPI vesicles are involved in retrograde transport from the Golgi to ER (Letourneur et al., 1994) and in budding from the ER (Bednarek et al, 1995). The third vesicle type carries proteins from the ER to the Golgi and is coated with a set of proteins known as COPII (Barlowe et al., 1994).

ER to Golgi Transport I: Role of the ER

The ER is the first compartment of the secretory pathway, and facilitates at least three processes critical for secretion: (1) folding of secretory proteins; (2) oligmerization of secretory proteins; (3) initial glycosylation of secretory proteins. Secretory proteins entering the ER encounter an environment very different than that of the cytosol; perhaps most

significantly, the ER is an oxidizing environment and facilitates the formation of disulfide bonds, while the cytoplasm is a reducing environment, and is not conducive to disulfide bond formation. The folding of secretory proteins in the ER is also facilitated by a number of resident ER proteins, including protein disulfide isomerase, which is thought to mediate disulfide bond rearrangement, and BiP, which appears to stabilize partially-folded intermediates (Freedman, 1984; Bole et al., 1986).

The oligomerization of many protein complexes also occurs in the ER; examples include the viral hemagglutinin trimer, the yeast V-ATPase protein complex, and the mammalian T-cell receptor complex (Doms et al., 1993; Bauerle et al., 1993; Bonifacino et al., 1989). Many proteins also receive glycolytic modifications en route through the secretory pathway, modifications which are usually initiated in the ER. Glycosylation has been shown to be important for intracellular protein folding and targeting, as well as for the stabilization of secreted proteins in the extracellular environment (Gahmberg and Tolvanen, 1996).

Following proper folding, oligomerization (if appropriate), and initial glycosylation in the ER, secretory proteins are transported to the *cis*-Golgi via membrane-enclosed vesicles, approximately 60 nm in diameter. 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 (Pelham, 1995), 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 in mammalian cells, at least some

secretory proteins are concentrated during ER to Golgi transport (Mizuno and Singer, 1993; Balch et al., 1994). 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 (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.

ER to Golgi Transport II: Experimental approaches

The dissection of ER to Golgi transport in yeast has been facilitated by both the discovery of conditional mutants that are defective in either vesicle formation or vesicle fusion, and by 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). Careful morphological analysis further divided the ER to Golgi transport-defective mutants into a class required for the formation of vesicles and a class 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 prepro-α-factor that has been post-translationally 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 mutation, antibody, or the absence of fusion

factors (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 Sec22, and Bos1p, but do not contain resident ER proteins (Barlowe et al., 1994; Rexach et al., 1994; Lian and Ferro-Novick, 1993). In addition, these vesicles have a characteristic set of twelve membrane proteins, termed ERV (ER-vesicle associated) proteins, one of which has been shown to be Emp24p/Bst2p (Rexach et al., 1994; Schimmöller et al., 1995).

ER to Golgi Transport III: COPII coat components

A. Overview

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). The COPII coat is morphologically distinct from the clathrin coat, and does not contain components of the coatomer (COPI) coats (Barlowe et al., 1994; Bednarek et al., 1994). In addition to these cytosolic factors, two additional proteins -- Sec16p and Sec12p -- 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). Sec16p binds to three cytosolic COPII coat components, Sec23p, Sec24p and Sec31p, and may be a foundation upon which these proteins assemble (Espenshade et al., 1995; Gimeno et al., 1996; Shaywitz et al., 1997). The integral ER membrane proteins Sec12p may regulate COPII vesicle formation, but is 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 depending upon the lysis conditions, these proteins can also be found associated with membranes in crude cell extracts. It is also noteworthy 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 initially escaped detection by the budding assay.

B. Sec23p/Sec24p

SEC23 was initially isolated as an ER to Golgi mutant in the original *sec* screen, and was subsequently determined to participate in vesicle formation (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; Yoshihisa et al., 1993); the precise stoichiometry of these two protein partners has not yet been established. Sec24p, like Sec23p, is essential, and is required for ER to Golgi transport both in vitro (Barlowe et al., 1994) and in vivo (R. Gimeno, A. Frand, and C. Kaiser, unpublished observations). Furthermore, the Sec23p/Sec24p complex represents one of the major components of the COPII vesicle coat, and is required for its formation (Barlowe et al., 1995; 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*. Sec16p has been shown to bind 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 (Gimeno et al., 1996). Sec31p also has been shown to bind directly to both Sec23p and Sec24p; for these interactions as well, the binding of one subunit to Sec31p does not require the presence of the other (Shaywitz et al., 1997). 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 hypothesis regarding Sec23p function (Oka and Nakano, 1994; Schekman and Orci, 1996), the role of the Sec23p/Sec24p complex in the formation of COPII vesicles still remains to be established.

Two human Sec23 homologs have been identified, hSec23Ap and hSec23Bp (Paccaud et al., 1996). The hSec23A protein complements a yeast *sec23* temperature sensitive mutant, and in mammalian cells, has been localized to the cisternae and vesicles in the region of the transitional ER. Gel filtration analysis of HepG2 cytosol suggests that hSec23Ap (like yeast Sec23p) fractionates as a 350 kD complex. Immunoprecipitation of hSec23A from clarified cytosol identifies a prominent band with an apparent molecular weight of 110 kD; this is approximately the molecular weight of yeast Sec24p. Although a mammalian Sec24p homolog has not yet been cloned, a cDNA encoding a protein with significant homolog to Sec24p has been reported (D38555; KIAA0079 gene product; Nomura et al., 1994).

C. Sec13p /Sec31p

SEC13 was isolated in the original screen for secretion mutants and 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 complex with the 150 kD Sec31p protein (Pryer et al., 1993; Salama et al, 1993; Salama et al., 1997). Although the size of the Sec31p/Sec13p complex was initially estimated to be approximately 700 kD, based on gel-filtration chromatography (Salama et al.,

1993), more recent studies using sedimentation through a glycerol gradient resulted in a size determination of 166 kD, approximately the predicted size of a Sec13p/Sec31p dimer (Salama et al., 1997). 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; Salama et al., 1997; A. Frand and C. Kaiser, unpublished observations). Structurally, Sec13p appears to consist almost entirely of six WD-40 repeats, a motif first described in the β-subunit of trimeric G-proteins, and subsequently shown to specify a β-propeller structure (Neer and Smith, 1996). Sec13p interacts with a WD-40-containing domain of Sec31p, raising the possibility of a homotypic interaction (Shaywitz et al., 1997). Sec13p also appears to interact with Sec16p (Appendix II), consistent with the proposed role for Sec16p in organizing COPII coat assembly (*vide infra*).

Further analysis of Sec31p has demonstrated that in addition to binding Sec13p, it can also bind Sec23p, Sec24p, and Sec16p (Shaywitz et al., 1997). While the N-terminal (WD-40) region of Sec31p appears to mediate binding to Sec13p, the extreme C-terminal region is required for binding to Sec16p. A Sec31p mutant lacking the final 127 amino acids of the 1273-amino acid protein can bind Sec13p, Sec23p, and Sec24p, but not Sec16p; this mutant also fails to complement a *sec31Δ* mutant, and cannot rescue the ER to Golgi transport defect of the temperature-sensitive *sec31-2* mutant. These data

suggest that the binding of Sec31p to Sec16p is essential, and required for ER to Golgi transport.

The precise 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 (*vide infra*). 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 Kar2p and Pdi1p 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).

Sec13p may also have functions apart from vesicle formation at the ER that are not shared by other COPII coat components. A role for Sec13p in the regulated transport of proteins from the Golgi to the plasma membrane was suggested by the sorting defect of the amino acid permease Gap1p observed in *sec13* mutant strains grown at temperatures permissive for ER to Golgi transport (Roberg et al., 1997). In addition, Sec13p and a Sec13p homologue, Seh1p, but not Sec31p are present in a subcomplex of the nuclear pore

(Siniossoglou et al., 1996). However, *sec13* mutants are not defective in nuclear import suggesting that the association of Sec13p with the pore may not be important for pore function. In contrast, Seh1p does appear to be involved in nuclear pore function, since *seh1* mutations genetically interact with other nuclear pore complex components (Siniossoglou et al., 1996).

A mammalian homolog of Sec13p, Sec13Rp, was discovered serendiptously (Swaroop et al., 1994). Although *SEC13R* itself could not complement a *sec13-1* mutant, chimeric constructs representing the N-terminal half of either Sec13p or Sec13Rp, and the C-terminal half of the other protein, could complement the *sec13-1* secretion defect (Shaywitz et al., 1995). Sec13p has also been shown to localize to the area of the transitional ER, in a distribution indistinguishable from that of mammalian Sec23 and Sar1. The localization of Sec13 was not affected by treatment with brefeldin A (BFA; an antibiotic that inhibits the nucleotide exchange activity required for the binding of ARF to membranes), in contrast to the localization of coatomer subunits; this insensitivity to BFA was shared by Sec23, and placed these two proteins in a different category from the proteins of the COPI complex. Finally, the ER to Golgi transport of the marker protein VSV-G was inhibited by anti-Sec13Rp antibodies in a semi-intact cell assay, providing further evidence that the function of Sec13 has been evolutionarily conserved (Tang et al., 1997). Although a mammalian homolog of Sec31p has not been identified, the finding that human Sec13Rp exhibits a strong and specific interaction with the N-terminal region of yeast Sec31p implies the existence of a mammalian Sec31 protein (Shaywitz et al., 1997).

D. Sec16p

SEC16 encodes an essential, 240 kD hydrophilic protein required for the formation of ER to Golgi transport vesicles in vivo (Kaiser and Schekman, 1990; Espenshade et al., 1995). Sec16p is tightly associated with the periphery of the ER, and is also found on ER-derived transport vesicles that are produced in vitro (Espenshade et al., 1995). In contrast to other COPII components, Sec16p cannot be extracted from membranes by urea, explaining why it has not needed to be added as a cytosolic factor to the in vitro transport assay (Espenshade et al., 1995). *SEC16* exhibits genetic interactions with the five other COPII genes (Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990; Gimeno et al., 1995) and Sec16p has been shown to bind directly to the COPII subunits Sec23p, Sec24p, and Sec31p (Espenshade et al., 1995; Gimeno et al., 1996; Shaywitz et al., 1997). In addition, membranes prepared from *sec16* mutant strains are defective for the formation of COPII vesicles in vitro (Cambell and Schekman, 1997); the in vitro formation of COPII vesicles is also inhibited by the addition of Sec16p-specific antibodies to the budding reaction (P. Espenshade, unpublished results). An attractive hypothesis is that Sec16p on the ER membrane serves as a COPII foundation, and organizes the recruitment and assembly of COPII coat components. This structure might be stabilized not only by the interaction of the two COPII complexes with Sec16p, but also by their interaction with each other (Shaywitz et al., 1997). Furthermore, the genetic interactions between *SEC16* and *SAR1* suggest that Sar1p could regulate the Sec16p-mediated coat assembly process (Nakano and Muramatsu, 1989, Gimeno et al., 1995).

A mammalian homolog of Sec16p has not been identified; a two-hybrid screen for such a protein is described in Chapter 3.

E. Sar1p

SAR1 was identified as a high-copy suppressor of mutations in *SEC12* and *SEC16* (Nakano and Muramatsu, 1989). *SAR1* is 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 components. 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 to increase the rate of guanine nucleotide exchange by 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 nonhydrolyzable 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 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 dissociation of Sec16p from the vesicle membrane ever occurs.

A mammalian homolog of Sar1p was identified using degenerate PCR (Kuge et al., 1995). Two clones, Sar1a and Sar1b were isolated; these clones are 91% identical to each other at the amino acid level, and are 61-67% identical to Sar1p. Immunoelectronmicroscopy using antibodies raised against Sar1a (and also reactive with Sar1b) demonstrated that Sar1, like mammalian Sec13 and Sec23, is found in the region of the transitional ER. Inhibition of the ER to Golgi transport of the marker protein VSV-G in vivo and in vitro using a deliberately designed dominant negative Sar1 mutant demonstrated the requirement for Sar1 in mammalian ER to Golgi transport.

F. Sec12p

Sec12p is an integral membrane protein that influences vesicle budding at the ER, but is not itself 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; Kaiser and Schekman, 1990; 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 may not 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 the ER membrane, Sec12p may effect recruitment simply by stimulating nucleotide exchange. 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 and Schekman, 1993).

ER to Golgi Transport IV: Cargo sorting during COPII vesicle formation

If important sorting decisions occur during the formation of COPII vesicles, as suggested above, 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-mediated 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., 1996; 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, an integral ER membrane protein, has been suggested to be such a receptor. Emp24p was cloned because it is also a prominent component of ER-derived vesicles

(Schimmöller et al., 1994); a chromosomal deletion of *EMP24* does not impair growth but slows ER to Golgi transport of a subset of cargo proteins (invertase and Gas1p), consistent with 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; *vide supra*, "Sec31p/Sec13p"). 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 secretion 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).

ER to Golgi Transport V: Towards a model for COPII vesicle formation

Biochemical and molecular analysis of the proteins required for vesicle formation suggest an outline for the pathway for COPII coat assembly. 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. This step is probably stimulated by Sec12p. Next, Sar1p in its GTP-bound, membrane-associated form may recruit the coat components Sec23p/Sec24p and Sec13p/Sec31p to Sec16p. This complex could then nucleate further

polymerization of coat subunits to eventually form a vesicle bud. This model is developed further in Chapter V.

ER to Golgi Transport VI: Future directions

The process of vesicle formation at the ER remains incompletely understood, and many fundamental questions remain. For example, how is the site of budding determined? What is the order of component assembly on the ER membrane? How is this assembly process regulated? Are there other factors required for vesicle assembly in vivo that, like Sec16p, have escaped detection by the in vitro assay? What is the relationship between the assembly of the coat structure and the deformation of the ER membrane from a flat surface into a vesicle bud? What is the mechanism of vesicle scission? What is the basis for cargo selection? What regulates dissociation of the coat? Resolution of these questions will require both the refinement of the in vitro assay as well as the isolation of additional mutants.

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Chapter II

Human SEC13Rp functions in yeast and is located on transport vesicles budding from the endoplasmic reticulum

PREFACE

This chapter has been published in its entirety in the Journal of Cell Biology as: David A. Shaywitz, Lelio Orci, Mariella Ravazzola, Anand Swaroop, and Chris A. Kaiser (1995): Human SEC13Rp functions in yeast and is located on transport vesicles budding from the endoplasmic reticulum. *J. Cell Biol.* 128:769-777.

A. Swaroop provided the human *SEC13R* clone; L. Orci and M. Ravazzola performed the immunoelectronmicroscopy.

ABSTRACT

In the yeast *S. cerevisiae*, Sec13p is required for intracellular protein transport from the endoplasmic reticulum (ER) to the Golgi, and has also been identified as a component of the COPII vesicle coat structure. Recently, a human cDNA encoding a protein 53% identical to yeast Sec13p has been isolated. In this report, we apply the genetic assays of complementation and synthetic lethality to demonstrate the conservation of function between this human protein, designated SEC13Rp, and yeast Sec13p. We show that two reciprocal human/yeast fusion constructs, encoding the N-terminal half of one protein and the C-terminal half of the other, can each complement the secretion defect of a *sec13-1* mutant at 36°C. The chimera encoding the N-terminal half of the yeast protein and the C-terminal half of the human protein is also able to complement a *SEC13* deletion. Overexpression of either the entire human SEC13Rp protein or the chimera encoding the N-terminal half of the human protein and the C-terminal half of the yeast protein inhibits the growth of a *sec13-1* mutant at 24°C; this growth inhibition is not seen in a wild-type strain nor in other *sec* mutants, suggesting that the N-terminal half of SEC13Rp may compete with Sec13-1p for a common target. We show by immunoelectronmicroscopy of mammalian cells that SEC13Rp -- like the putative mammalian homologues of the COPII subunits Sar1p and Sec23p -- resides in the region of the transitional ER. We also show that the distribution of SEC13Rp is not affected by brefeldin A treatment. This report presents the first demonstration of a putative mammalian COPII component functioning in yeast, and highlights a potentially useful approach for the study of conserved mammalian proteins in a genetically tractible system.

INTRODUCTION

Proteins secreted by eukaryotic cells are vectorially transported from the ER through the Golgi apparatus to the cell surface in a series of steps mediated by membrane-bounded vesicles (Palade, 1975). The ability to study vesicle budding and fusion events in both yeast and mammalian systems has greatly facilitated the detailed molecular study of intracellular protein transport (Pryer et al., 1992; Rothman and Orci, 1992; Kaiser, 1993).

Vesicular transport between adjacent Golgi stacks has been extensively investigated in mammalian systems, and a clear model has now emerged (Ostermann et al., 1993). In this scheme, transport is initiated by the attachment of the small molecular weight GTP-binding protein, ADP-ribosylation factor (ARF), to the donor compartment (Donaldson et al., 1992; Helms and Rothman, 1992; Helms et al., 1993). Bound ARF-GTP stimulates the recruitment of coatomer, a seven-subunit protein complex, from the cytosol, and budding occurs when coatomer binds (Donaldson et al., 1992; Orci et al., 1993b,c; Palmer et al., 1993; Hara-Kuge et al., 1994). Finally, the hydrolysis of ARF-GTP, presumably at the target membrane, results in coat disassembly, and permits the vesicle to fuse (Tanigawa et al., 1993; Elazar et al., 1994).

Both ARF and the β-COP subunit of coatomer have also been implicated in ER to Golgi transport. Transport of the vesicular stomatitis virus glycoprotein (VSV-G) from the ER is inhibited by the overexpression of a dominant negative ARF mutant (T31N) in cultured cells (Dascher and Balch, 1994). VSV-G transport from the ER is also inhibited by β-COP-specific antibodies in both microinjected (Pepperkok et al., 1993) and digitonin-

permeabilized (Peter et al., 1993) cells . These data suggest a possible role for coatomer in ER to Golgi transport.

Vesicular transport from the ER to the Golgi has been extensively studied in the yeast *S. cerevisiae*, and more than 20 genes have been implicated in this process (Kaiser, 1993). One of these genes, *SEC21*, is homologous to the γ-subunit of coatomer (Hosobuchi et al., 1992). However, purification of *S. cerevisiae* transport-competent vesicles synthesized *in vitro* has revealed a coat complex containing five proteins previously implicated in vesicle-formation -- Sec13p, Sec23p, Sec24p, Sec31p and Sar1p -- but not Sec21p (Barlowe et al., 1994). Sec13p and Sec23p were initially identified by temperature-sensitive mutations which block vesicle formation at the non-permissive temperature (Novick et al., 1980; Kaiser and Schekman, 1990). Sec24p and Sec31p were identified by the physical association with Sec23p and Sec13p, respectively (Hicke et al., 1992; Salama et al., 1993). Sar1p was initially isolated as a suppressor of a mutation in the vesicle formation gene *SEC12* (Nakano and Muramatsu, 1989). Since none of these proteins show any apparent homology to subunits of the mammalian coatomer, the yeast coat complex has been designated COP II (Barlowe et al., 1994).

Mammalian genes showing significant sequence similarity to three COPII proteins have now been identified. Using degenerate PCR, Kuge et al. (1994) isolated two different mammalian clones encoding proteins with predicted amino acid sequences 61% identical to the sequence of the yeast Sar1p protein. The export of VSV-G protein from the ER is inhibited by overexpression of a dominant negative Sar1p mutant (T39N), as well as by the incubation of semi-intact cells with Sar1p-specific antibody. Immuno-electronmicroscopy (EM) analysis reveals that Sar1p is highly enriched on vesicular carriers in the transitional region of the ER, consistent with a role in

ER to Golgi transport. A similar distribution was also seen in mammalian cells for Sec23p, using cross-reacting antibodies raised against the yeast Sec23p protein (Orci et al., 1991). A mouse Sec23p homologue, encoding a product 40% identical to Sec23p, was recently discovered (Wadhwa et al., 1993); the relationship between this protein and the protein that cross-reacts with the anti-yeast-Sec23p antibody has not yet been established. Finally, Swaroop et al. (1994) have identified a human gene, *SEC13R*, that encodes a protein with 53% identity and 70% similarity to the amino acid sequence of the yeast COPII protein Sec13p.

The extensive genetic study of secretion provides us with the tools to explore the relationship between the function of a yeast COPII component and the function of a potential mammalian homolog. Perhaps the most direct way to study this relationship is to examine the behavior of the human protein in yeast -- particularly yeast bearing a mutation in the corresponding endogenous gene. Not only is this approach useful in helping us understand and compare the process of vesicular transport in yeast and mammalian cells, but evidence of interchangability is also required for the rigorous demonstration of homology (Tugendreich et al., 1994).

To investigate the functional relationship between the human protein SEC13Rp and yeast Sec13p, we examined the effect of human *SEC13R* expression in mutant and wild-type yeast. Through the application of two different genetic criteria -- complementation (Benzer, 1962) and synthetic lethality (Dobzhansky, 1946; Sturtevant, 1956; Huffaker, 1987) -- we have been able to show that *SEC13R* exhibits *SEC13* function, and may therefore be designated a mammalian homolog of *SEC13*. Although *SEC13R* itself does not complement the temperature-sensitive yeast mutant *sec13-1*, two reciprocal human/yeast chimeras, encoding the N-terminal of one protein

and the C-terminal half of the other, are each able to rescue the *sec13-1* secretion defect at 36°C; one of the chimeras can also complement a *SEC13* deletion. Furthermore, overexpression of the entire mammalian gene exhibited a negative effect in *sec13-1* mutants but not in either wild-type yeast or in other *sec* mutants; this result, an example of the genetic phenomenon of synthetic lethality, strongly suggests that in yeast, Sec13p and SEC13Rp participate in the same pathway. Together, the genetic data provide compelling evidence that human SEC13Rp can function in yeast; SEC13Rp thus represents the first putative mammalian homolog of a yeast COPII component to fulfill this criterion.

Immuno-EM analysis of pancreatic cells using antibodies raised against SEC13Rp demonstrate that SEC13Rp is concentrated in the transitional ER, in a distribution indistinguishable from that previously observed for Sec23 in the same cell type (Orci et al., 1991; Orci et al., 1993a). The distribution of SEC13Rp is not affected by brefeldin A treatment, in contrast to the result seen for coatomer (Orci et al., 1993a).

As a whole, our data not only support the existence of a mammalian COPII structure, but also emphasize the utility of heterologous gene expression as tool to study conserved proteins (Whiteway et al., 1993; Thukral et al. 1993).

MATERIALS AND METHODS

Strains, Materials, and General Methods

Yeast strains used in this study are CKY8 (*MATα leu2-3,112 ura3-52*), RHY305 (*MATα leu2-3,112 ura3-52 sec13-1* GAL), DSY174 (*MATa leu2-3,112 ura3-52* GAL [pRS315, pGAL-SEC13R]), DSY223 (*MATα sec12-4 leu2-3,112 ura3-52* GAL [pRS315, pGAL-SEC13R]), DSY216 (*MATα sec13-1 leu2-3,112 ura3-52* GAL[pRS315, pGAL-SEC13R]), DSY228 (*MATa sec16-2 leu2-3,112 ura3-52* GAL[pRS315, pGAL-SEC13R]), DSY232 (*MATa sec17-1 leu2-3,112 ura3-52* GAL [pRS315, pGAL-SEC13R]), DSY236 (*MATa sec18-1 leu2-3,112 ura3-52* GAL [pRS315, pGAL-SEC13R]).

Yeast culture, genetic manipulations, and molecular techniques were as described (Sambrook et al., 1989; Rose et al., 1990). Mammalian cell extracts were prepared by standard methods (Harlow and Lane, 1988). Materials were obtained from Sigma Chemical Company (St. Louis, MO) unless stated otherwise.

Gel electrophoresis was performed according to the Laemmli SDS-PAGE method using 10% polyacrylamide (Laemmli, 1970). Affinity purified anti-SEC13Rp antibody was used at 1:10,000 dilution. Secondary antibody was goat-anti-rabbit IgG, conjugated to HRP (Amersham Corp., Arlington Heights, IL), at a 1:10,000 dilution. Filter-bound antibodies were then detected by peroxidase-catalyzed chemiluminescence (ECL kit, Amersham Corp.).

The monkey fibroblast cell line COS was grown in complete medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. The chinese hamster ovary cell line CHO was grown in complete medium consisting of Ham's F12 medium with 5% fetal bovine serum, 2 mM

glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Both cells lines were maintained at 37°C in a 5% CO_2 cell incubator.

Generation of Chimeric Constructs

The cloning of the *SEC13R* gene has been previously described (Swaroop et al., 1994). A ClaI site was introduced at nucleotide 449 in the *SEC13R* cDNA, corresponding to the ClaI site present in *SEC13* at nucleotide 488 (Pryer et al., 1993), using the oligonucleotide-directed mutagenesis method of Kunkle et al. (1987). The (antisense) primer used for this mutagenesis was: 5'-GGCAATGGTGTGAGCATCGATGATCTTCTTTACTTC-3'; altered nucleotides are underlined. In addition to introducing a restriction site, this procedure also altered two amino acids, changing Asn146 -> Ile and Asn147 -> Asp (Fig. 1). Both mutagenized and wild-type forms were cloned into the vector pCD43 directly downstream of the *GAL10* promoter. Since both forms behaved identically in all assays described, only the strain containing the mutagenized form (designated pGAL-SEC13R) is shown. pCD43 is a modified pRS316 (*ARS CEN URA3*) vector (Sikorski and Hieter, 1989) in which divergent *GAL1* and *GAL10* promoters have been introduced between the BamHI and EcoRI sites in the polylinker. The N-terminal Sec13p/C-terminal SEC13Rp chimera was constructed by linking a BamHI/ClaI *SEC13* 5' fragment with a ClaI-HindIII *SEC13R* 3' fragment. The reciprocal chimera was constructed by linking a 5' RI-ClaI *SEC13R* fragment with a ClaI-KpnI *SEC13* fragment. The *SEC13* plasmid was generated by first introducing a BamHI site immediately 5' to the initial ATG by PCR, using pCK1313 as a template (Pryer et al., 1993) and 5'-GCGGATCCAACCATGGTCGTCATAGCTAATGC-3' as the (sense) primer.

The resulting BamHI/SacI fragment was cloned into pCD43, and was able to rescue *sec13-1* mutants at the restrictive temperature.

Chimera Complementation/Inhibition Assays

All constructs were transformed into RHY305. The vector pRS315 (Sikorski and Hieter, 1989) was co-transformed in all experiments, rendering all strains effectively prototrophic. Transformants were then assayed at 36°(complementation) or at 24°(inhibition) on synthetic minimal media (Difco) supplemented with either 2% glucose or 2% galactose.

Radiolabelling and Immunoprecipitations

Cells were pre-grown at 24°C in selective SC medium containing 2% raffinose, and were induced by the addition of 2% galactose three hours prior to the start of labeling. One hour before labelling, cells were shifted to 36°C. $8x10^7$ exponentially growing cells (4 OD_{600} units) were radiolabelled in supplemented SD medium by incubating with 30 µCi ^{35}S-methionine per OD_{600} unit (Express protein labelling mix [NEN, Boston, MA], spec. activity 1200 Ci/mmol). Samples were chased by the addition of 1/100 volume of a solution containing 0.1M ammonium sulfate, 0.3% cysteine, 0.4% methionine. Labelled samples of 1 OD_{600} unit of cells were collected into chilled tubed containing one volume of 40 mM sodium azide. Protein extracts were prepared in 30 ul ESB by vigorous agitation with glass beads. Extracts were diluted with 1 ml IP buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.2% SDS), absorbed for 20 minutes with 50 ul 10% *S. aureus* cells (Sigma), and cleared by centrifugation at 12,000 g for five minutes. 0.5 ul anti-CPY antibody was added, and extracts were rotated for one hour at room temperature. Immune complexes were collected by adding 25 ul 50%

Protein A/Sepharose (Pharmacia, Piscataway, NJ) per sample, and incubating an additional hour at room temperature. Protein A/Sepharose pellets were washed twice with IP buffer, and twice with detergent-free IP buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl). Protein was released into 30 ul ESB by heating to 100°C for 2 minutes. 12 ul of the supernatant were separated by SDS-PAGE and visualized by fluorography (Harlow and Lane, 1989).

Generation of *SEC13*-deletion strain

RHY297 (*MATα sec13-Δ1 ura3-52 leu2-3,112 ade2-101 ade3-24* [pKR4 (*ARS CEN LEU2 ADE3 SEC13*)]) carries a *sec13* allele (*sec13-Δ1*) in which the entire *SEC13* coding sequence is deleted. RHY297 was constructed by R. Hammer as follows. A 50-nucleotide oligomer (SEC13-N1: 5'-CAT TTT AAA TTC TTG ATA CTC TTC ACG GAT CCG TAT GCT GAT ATA AAA TTA TCT GTT ATC-3') consisting of 28 and 26 nucleotides complementary to the 5' and 3' untranslated region of *SEC13* respectively and 6 nucleotides creating a BamHI site (underlined) was used to mutagenize pKR1 (*CEN SEC13 URA3*) (K. Roberg, unpublished) using the protocol of Kunkel et al. (1987). pRH102 is a mutagenesis product that lacks the entire *SEC13* coding region as judged by restriction mapping, but retains *SEC13* flanking DNA. A 5 kb *hisG-URA3-hisG* marker cassette (modified Alani et al. [1987]); S. Elledge, unpublished) was inserted into the BamHI site of pRH102 to make pRH104. The 6kb EcoRI-SacI fragment of pRH104 was transformed into KRY5p4 (*MATα sec13-1 ura3-52 leu2-3,112 ade2-101 ade3-24* [pKR4 (*ARS CEN LEU2 ADE3 SEC13*)]) (K. Roberg unpublished). Ura^+ transformants that are unable to sector at 24°C were selected. RHY297 is a non-sectoring, Ura^- derivative, selected by plating transformants on medium containing 5-fluoro-orotic acid (Boeke et al., 1984).

The chimera-suppressed *SEC13*-deletion strain was obtained by transformation of RHY297 using the construct encoding the N-terminal Sec13p/C-terminal SEC13Rp chimera, described above, and then isolating white (pKR4$^-$) colonies.

Generation of Antibodies to the SEC13Rp Protein

SEC13Rp antiserum was elicited against a hybrid protein composed of the entire *SEC13R* coding region fused to Staphylococcal protein A, using the pRIT33 vector (Nilsson and Abrahmsen, 1990). Hybrid protein was prepared from *E. coli* extracts and antibody to this protein produced in rabbits, as described previously (Griff et al., 1992). Antiserum was affinity purified using a ß-galactosidase-SEC13Rp hybrid protein constructed by fusing the entire coding sequence of *SEC13R* to the *lacZ* gene in the pEX2 vector (Stanley and Luzio, 1984). The hybrid protein was isolated and used for affinity purification of the antibody as described (Pryer et al., 1993). The affinity-purified anti-sera was concentrated using a centricon-30 microconcentrator (Amicon).

Immunofluorescence

COS cells or CHO cells were grown in complete medium on 12 mm glass coverslips for 2 days prior to recovery. Cells were fixed in 2% paraformaldehyde, and permeabilized in 0.1% Triton X-100, 0.02% SDS. Affinity-purified anti-SEC13Rp was used at a dilution of 1:50 in PBS/10% FBS in the presence of 0.2% saponin; 2° antibody (FITC-conjugated goat-anti-rabbit; Boehringer) was added at a concentration of 1:250, using the same buffer composition. Coverslips were viewed by epifluorescence with a Zeiss Axioskop.

Immunoelectronmicroscopy

Rat pancreatic acinar tissue and isolated islets of Langerhans fixed in 1%, phosphate-buffered glutaraldehyde were processed for cryoultramicryotomy according to Tokuyasu (Tokuyasu, 1986). SEC13Rp was localized by the protein A-gold method (Roth et al., 1978). Antibody dilution = 1:50; gold particle size = 10 nm. After immunolabeling, the cryosections were stained with uranyl acetate. Brefeldin A (BFA) treatment of insulin cells was performed as previously described (Orci et al., 1993a).

RESULTS

Complementation of *sec13-1* defect by human/yeast chimeras

The high degree of sequence similarity between Sec13p and SEC13Rp (Fig. 1), distributed along the length of the two proteins, encouraged us to ask whether *SEC13R* could functionally substitute for a defective *SEC13* gene. The *sec13-1* allele represents a single point mutation in *SEC13*, and cannot support growth at temperatures above 30°C (Pryer et al., 1993). The overexpression of *SEC13R* cDNA from a galactose-inducible promoter in a *sec13-1* strains did not restore viability at 36°C (Fig. 2a). However, significant growth was observed at 36°C upon the galactose-induced overexpression of a chimeric construct encoding the N-terminal half of yeast Sec13p and the C-terminal half of human SEC13Rp. This chimera was constructed by first creating a ClaI restriction site at nucleotide 449 of the human cDNA, corresponding to a naturally occurring ClaI site in nucleotide 488 of the yeast gene; each ClaI site occurs roughly in the middle of the protein coding sequence. The N-terminal-encoding half of yeast *SEC13* was then fused to the C-terminal-encoding half of human *SEC13R*, using the ClaI site as a junction (Fig. 1). Induction of a construct encoding only the N-terminal half of yeast Sec13p was unable to complement a *sec13-1* defect (data not shown; Pryer et al., 1993), suggesting that the C-terminal region of Sec13p is functionally required, and is not dispensable (see Discussion). The galactose-induced overexpression of the reciprocal chimeric construct, encoding the N-terminal half of human SEC13Rp and the C-terminal half of yeast Sec13p, also complemented the *sec13-1* defect, though somewhat less efficiently (Fig. 2a).

To demonstrate that both chimeras correct the *sec13* secretion defect, the transport of the marker protein carboxypeptidase Y (CPY) was monitored by pulse-chase analysis. CPY is targeted to the vacuole via the ER and Golgi. The core-glycosylated p1 form of the enzyme can be resolved from the form that has received Golgi-specific modification (p2) and the mature form (M) that has been proteolytically cleaved in the vacuole (Stevens et al. 1982). Cells were grown in exponential phase at 24°C in media containing raffinose, induced for 2 hours with galactose, then shifted to 36°C for one hour. Cells were next pulse-labeled for 10 minutes, then chased with excess methionine and cysteine. Lysates were prepared, and immunoprecipitated with anti-CPY antibodies. In *sec13-1* mutants, at 36°C, CPY is unable to exit the ER, and exists almost exclusively in the p1 form (Fig. 3). However, upon the galactose-induced overexpression of either the N-terminal yeast Sec13p/C-terminal human SEC13Rp chimera or the N-terminal human SEC13Rp/C-terminal yeast Sec13p chimera, CPY is able to exit the ER, progress through the Golgi, and arrive in the vacuole (Fig. 3). The observation that either half of SEC13Rp can supply sufficient Sec13p activity to permit a yeast/human chimeric protein to complement the temperature-sensitive secretion defect of a *sec13-1* mutant suggests that SEC13Rp and Sec13p are functionally similar.

The ability of SEC13Rp to exhibit Sec13p function was illustrated further by the viability of a yeast strain expressing an N-terminal yeast Sec13p/C-terminal human SEC13Rp chimeric protein, but containing no endogenous Sec13p (Fig. 4). To determine whether a yeast/human chimera could complement a *SEC13* deletion, the N-terminal Sec13p/C-terminal SEC13Rp construct was transformed into the indicator strain RHY297. RHY297 is an *ade2- ade3-* strain in which the entire *SEC13* coding sequence has been deleted; the strain carries a plasmid bearing *SEC13, LEU2* and *ADE3*.

The strain is normally dependent upon the plasmid-encoded *SEC13*, and is also colored, reflecting the accumulation of a red intermediate resulting from *ade2*-blocked adenine biosynthesis (Jones and Fink, 1981; Koshland et al., 1985). However, upon transformation with a plasmid capable of complementing the *SEC13* deletion, the *SEC13, ADE3, LEU2* plasmid is no longer required, and can be lost during colony growth. Plasmid loss is detected by the appearance of white sectors; in the absence of the *ADE3* gene product, the adenine biosynthetic pathway is blocked at an earlier stage, prior to the *ade2* block, and the red-colored intermediate is not produced.

RHY297 was transformed with the construct encoding the N-terminal Sec13p/C-terminal SEC13Rp chimeric protein, grown on plates containing galactose but not leucine (to maintain selection for the *LEU2* marker), and analyzed by western blot using affinity-purified anti-Sec13p antibodies as a probe (Pryer et al., 1993). Yeast Sec13p itself has a predicted molecular weight of approximately 33 kD. The yeast/human chimera, however, has a slightly heavier predicted molecular weight, since the human C-terminal region is 20 amino acids longer than the corresponding region of the yeast protein. Extracts from wild-type cells revealed a single band at 33 kD, representing endogenous Sec13p (Fig. 3). Extracts from RHY297 cells transformed with the yeast/human chimeric construct revealed two bands: a 33 kD band representing Sec13p, and a slower-migrating band representing the chimeric construct.

When the RHY297 strain containing the chimeric construct was then grown on plates containing galactose plus rich media (thus no longer maintaining selection for the *LEU* marker on the *SEC13* plasmid), white sectors appeared, indicating that in the presence of the chimeric construct, the Sec13p-encoding plasmid was no longer necessary. When colonies from the

white sectors were isolated and analyzed by western blot, a single band appeared at the location expected for the chimeric protein (Fig. 4). These data demonstrate that the N-terminal Sec13p/C-terminal SEC13Rp chimeric protein can functionally substitute for an absent Sec13p protein. Furthermore, since the chimeric product can functionally replace the endogenous protein, the *SEC13R*-encoded C-terminal region must exhibit activity similar to that found in the C-terminal region of Sec13p. Complementation of a *SEC13* deletion was not noticeably detected in a yeast strain transformed with the reciprocal chimera, a result consistent with the weaker activity exhibited by this construct in the suppression of a *sec13-1* growth defect (Fig. 2a).

Human SEC13R specifically inhibits growth of a *sec13-1* mutant

In the course of the complementation experiments, we discovered that the galactose-induced overexpression of SEC13Rp inhibited the growth of a *sec13-1* mutant strain at permissive temperatures. Although this strain normally grows well at 24°C, cell growth was dramatically inhibited by the induction of SEC13Rp (Fig 2b). Galactose-induced overexpression of SEC13Rp produced no evident phenotype in a wild-type strain or in strains carrying temperature-sensitive alleles of *SEC12*, *SEC16*, *SEC17*, or *SEC18*, suggesting that the observed effect was specific for the *sec13-1* strain (Fig. 5). Overexpression of the chimera encoding the N-terminal half of SEC13Rp and the C-terminal half of Sec13p also inhibited growth of the *sec13-1* strain at 24°C (Fig. 2b), implying that the N-terminal half of SEC13Rp was responsible for this growth inhibition. Consistent with this interpretation, the N-terminal Sec13p/C-terminal SEC13Rp chimera produced no obvious phenotype at 24°C. The inhibitory effect of SEC13Rp expression on the

growth of a *sec13-1* strain at 24°C suggests that both SEC13Rp and Sec13-1p may interact with the same protein or substrate, and thus may participate in the same step or pathway. For example, SEC13Rp may titrate out a Sec13p target by binding to it unproductively.

Subcellular localization of SEC13Rp

To explore further the function of SEC13Rp in mammalian cells, we raised rabbit serum against a hybrid protein of *Staphylococcus* protein A fused to SEC13Rp. Antibodies specific for SEC13Rp were affinity purified using a hybrid protein composed of *E. coli* β-galactosidase fused to SEC13Rp. Purified antibodies recognized a single prominent band with the expected molecular weight of approximately 36 kD on immunoblots of lysates from wild-type yeast overexpressing SEC13Rp or from CHO or COS cells (Fig. 6). Immunoblots of wild-type yeast lysates did not show any pronounced bands; this shows that the affinity-purified anti-SEC13Rp antibodies do not cross-react with the yeast Sec13p protein.

The affinity-purified antibody was used to determine the subcellular localization of SEC13Rp. By indirect immunofluorescence, SEC13Rp exhibited a "diamond ring" appearance, encircling the nucleus in both CHO and COS cells (Fig. 7a, b). To determine more precisely the subcellular distribution of SEC13Rp, we used immunoelectronmicroscopy, and focused on two cell types with well-developed secretory compartments, the insulin and the acinar cells of the pancreas (Fig. 8a, b). In both of these cell types, SEC13Rp was found concentrated in the transitional area of the ER (Table 1), in a distribution indistinguishable from that previously observed for mammalian Sec23p in the same cells (Orci et al., 1991). Furthermore, as previously observed for mammalian Sec23p (Orci et al., 1993a), the

distribution of SEC13Rp was not altered by brefeldin A treatment, and remained excluded from coatomer-rich areas of "BFA-bodies" (Fig. 9). These data are consistent with the view that SEC13Rp and mammalian Sec23p are not constituents of the coatomer, but rather are both components of a different structure -- most likely, mammalian COPII -- involved in ER to Golgi transport.

DISCUSSION

This report provides strong evidence that the function of Sec13p has been highly conserved through evolution. In yeast, Sec13p is required for vesicle budding from the ER, and has been identified as a constituent of the vesicle-coating protein complex designated COPII (Pryer et al., 1993; Barlowe et al., 1994). We have shown that two reciprocal human/yeast chimeric Sec13 constructs can each complement a *sec13-1* mutant, and can rescue both the growth defect and the secretion defect. We have also shown that one of these chimeric constructs can complement a deletion of *SEC13*. In addition, we have demonstrated that the human SEC13Rp protein itself inhibits the growth of a *sec13-1* mutant, but not of either wild-type yeast or mutants in *SEC12*, *SEC16*, *SEC17*, or *SEC18*. We also show that SEC13Rp is located in the transitional ER, in a distribution identical to that previously observed for mammalian Sec23. Finally, we show that the cellular distribution of SEC13Rp is insensitive to BFA treatment, consistent with the behavior previously observed for mammalian Sec23, but not for coatomer components.

Complementation of the growth defect of a *sec13-1* mutant at 36°C represents a stringent assay that requires no presumptions about the specific function of Sec13p. The ability of both yeast/human chimeric proteins to complement a *sec13-1* mutant implies that in both cases, the human region of the chimeric protein is fulfilling the function or functions normally performed by the corresponding region of the yeast protein. The ability of both chimeras to restore CPY transport in a *sec13-1* strain specifically

demonstrates complementation of the ER to Golgi secretion defect characteristic of *sec13-1* mutants.

If the two reciprocal chimeras complement both the growth defect and the secretion defect of a *sec13-1* mutant, then why does the entire *SEC13R* cDNA not complement either defect? We believe that there are at least two contributing factors. First, we would suggest that although both halves of human SEC13Rp can function in yeast, they do not function at quite the same level of wild-type Sec13p; this would explain why the full-length SEC13Rp protein, representing the sum of two sub-optimal halves, cannot rescue the *sec13-1* secretion defect. Second, we would propose that in a *sec13-1* strain, the N-terminal region of SEC13Rp is toxic, resulting in the growth defect observed at 24°C in strains expressing either the full length SEC13Rp protein or the N-terminal SEC13Rp/C-terminal Sec13p protein. Although the precise reason for this toxicity is not known, the observation that it is only seen in a *sec13* mutant, and not in either a wild-type strain or in other *sec* mutants, suggests a synthetic lethal interaction between *SEC13R* and *sec13-1*, and implies that sec13-1p and SEC13Rp participate in the same pathway, and perhaps compete for a common target. The ability of the N-terminal SEC13Rp/C-terminal Sec13p protein to rescue a *sec13-1* secretion defect at 36°C suggests that the toxicity associated with the N-terminal region of SEC13Rp develops over a period of time longer than that encompassed by the pulse-chase assay.

Localization of SEC13Rp to the transitional ER and associated transport vesicles represents an important observation. These data not only situate SEC13Rp in precisely the area expected for a mammalian protein involved in ER to Golgi transport, but also emphasize that SEC13Rp is concentrated at this level. This is strong evidence that SEC13Rp is specifically involved in ER to

Golgi transport. If SEC13Rp is in fact a component of a mammalian COPII complex, then these data would suggest that COPII is involved solely in transport between the ER and the Golgi, and, unlike coatomer, is not involved in intra-Golgi transport.

The observation that SEC13Rp does not redistribute upon BFA treatment is encouraging because it places SEC13Rp and mammalian Sec23p in a different category from all the known coatomer components. Furthermore, since BFA is known to inhibit the binding of ARF to donor membranes, it is tempting to speculate that ARF is not involved in the recruitment of SEC13Rp and mammalian Sec23p; perhaps this function is fulfilled by Sar1p.

Functional complementation of a yeast mutant by a human/yeast chimera has been reported for a number of different genes. Mutants in yeast genes encoding the transcription factor SWI2p (Khavari et al., 1993), the nucleotide exchange factor CDC25p (Wei et al., 1992), the RNA-binding protein SNP1p (Smith and Barrell, 1991), and the ABC-transporter STE6p (Teem et al., 1993) have all been complemented by chimeric constructs consisting of fused regions of the wild-type yeast gene and its putative mammalian homologue. Not only does such complementation demonstrate the conservation of function between yeast and human gene products, but it can also allow for the detailed study of the mammalian protein, as is illustrated by the work by Teem et al. (1993) on the cystic fibrosis transmembrane conductance regulator.

Together, the data presented in this report argue that the function of Sec13p has been conserved from yeast to humans, and also support the existence of a mammalian COPII complex. More generally, our results emphasize that chimera studies represent a useful, easily-adaptable approach

for applying the tools of yeast genetics to the study of conserved mammalian proteins.

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Table I

SEC13Rp immunogold labeling of transitional area, ER, and Golgi of pancreatic acinar and insulin cells

	No. of gold particles per μm^2 ± SEM	
	Acinar cell	Insulin cell
ER (n=10)	0.34	0.18
Transitional area* (n=10)	61± 13 (54 ±6 vesicles per μm^2; 28% ± 4% of vesicles labeled)	110 ± 23 (105 ±15 vesicles per μm^2; 48% ±7% of vesicles labeled)
Golgi (n=10)	1±0.7	2±1

n = number of pictures evaluated. Quantitation was performed as described previously (Orci et al., 1991).

* Including the budding front of the transitional ER, vesicles, and intervening cytosol.

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FIGURES

Figure 1

Sequence comparison of yeast Sec13p and human SEC13Rp. Identities are indicated by solid lines, similarities are indicated by dotted lines. The two amino acids altered during site-directed mutagenesis, corresponding to the junction site for chimera construction (see Methods), are indicated by carats. The Sec13p amino acids altered in the three known *SEC13* temperature-sensitive alleles (*sec13-1*, *sec13-4*, *sec13-5*) are underlined (Pryer et al., 1993).

```
ın SEC13Rp   1 MVSVINTVDTSHEDMIHDAQMDYYGTRLATCSSDRSVKIFDVRNGGQILI 50
               || : |     |:::|||| :|||| |||||||: :|||:|  : : ||
ıst Sec13p   1 MVVIAN....AHNELIHDAVLDYYGKRLATCSSDKTIKIFEVEGETHKLI 46

            51 VDLRGHEGPVWQVAWAHPMYGNILASCSYDRKVIIWREENGTWEKSHEHA 100
               | ||||||| | |||| :| |||||||| ||:||:|||| |      ||
            47 DTLTGHEGPVWRVDWAHPKFGTILASCSYDGKVLIWKEENGRWSQIAVHA 96

           101 GHDSSVNSVCWAPHDYGLILACGSSDGAISLLTYTGEGQWEVKKINNAHT 150
               |  ||||| ||||:|| :|  :|||| :|:: : ::|       | :||
            97 VHSASVNSVQWAPHEYGPLLLVASSDGKVSVVEFKENGT.TSPIIIDAHA 145
                                                          ^^
           151 IGCNAVSWAPAVVPGSLIDHPSGQKPNYIKRFASGGCDNLIKLWKEEEDG 200
               || |  ||||| : :   :   | |    ::|  || |||:|:|| : |:
           146 IGVNSASWAPATIEED..GEHNGTKES..RKFVTGGADNLVKIWKYNSDA 191

           201 Q.WKEEQKLEAHSDWVRDVAWAPSIGLPTSTIASCSQDGRVFIWTCDDAS 249
               | :  |  ||:|||||||||| | : |  | :|| |||   :||| :|
           192 QTYVLESTLEGHSDWVRDVAWSPTVLLR.SYLASVSQDRTCIIWT.QDNE 239

           250 SNTWSPKLL..HKFNDVVWHVSWSITANILAVSGGDNKVTLWKESVDGQW 297
                  |   ||   || ||:|: |||: :|:||:|||||||||||| ::| |
           240 QGPWKKTLLKEEKFPDVLWRASWSLSGNVLALSGGDNKVTLWKENLEGKW 289

           298 VCISDVNK 305
                  ::|:
           290 EPAGEVHQ 297
```

Figure 1

Figure 2

Effect of human/yeast chimeras in a *sec13-1* mutant strain. (a) Chimeras between human *SEC13R* and yeast *SEC13* can complement a *sec13-1* temperature-sensitive allele at the non-permissive temperature (36°C). *SEC13R*, *SEC13*, or chimeric constructs were cloned downstream of a galactose-inducible promoter in a centromeric vector, transformed into a *sec13-1* mutant, and assayed for growth in the presence of galactose (induced) or glucose (uninduced) at non-permissive conditions (36°C). (b) SEC13Rp overexpression inhibits the growth of a *sec13-1* strain. Assay performed at 24°, but otherwise conditions identical to (a).

Figure 2

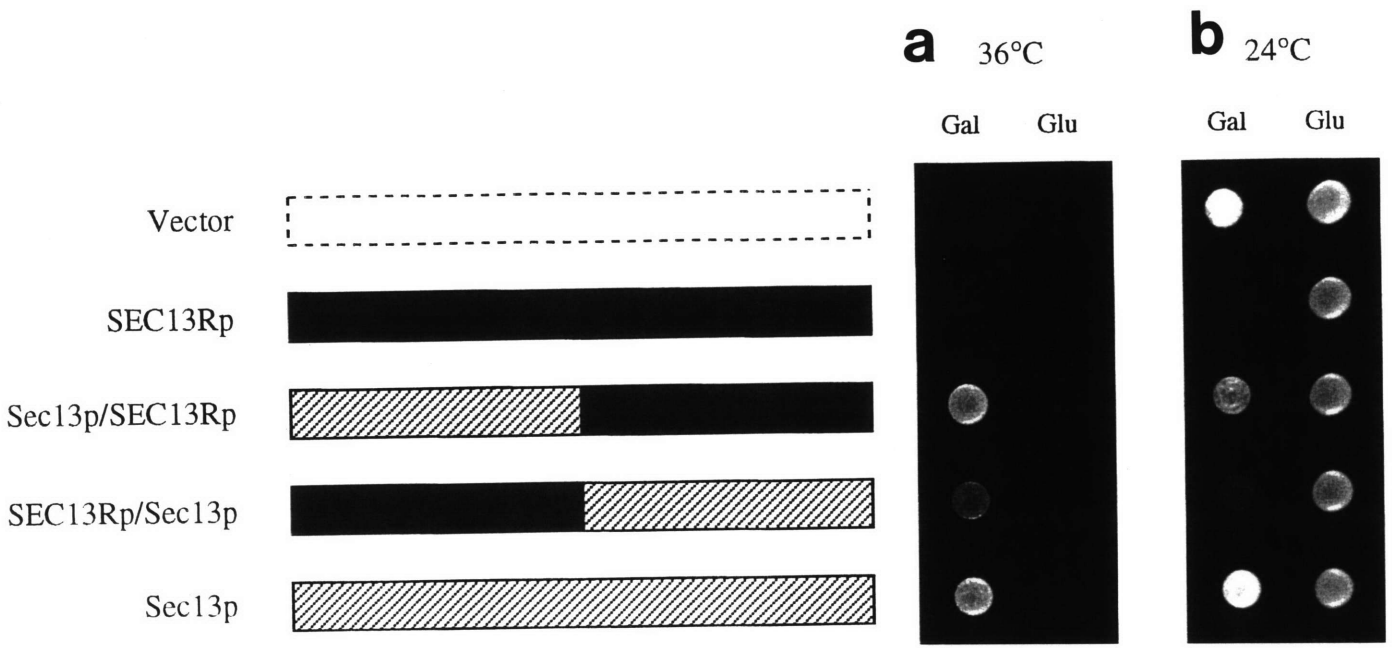

a 36°C
b 24°C
Gal
Glu
Gal
Glu
Vector
SEC13Rp
Sec13p/SEC13Rp
SEC13Rp/Sec13p
Sec13p

Figure 3

Complementation of the *sec13-1* secretion defect by both Sec13p/SEC13Rp and SEC13Rp/Sec13p chimeras. *sec13-1* cells carrying the indicated plasmid were induced for 2 hours in galactose at 24°C, shifted to 36°C for one hour, pulse-labeled with Tran^{35}S-label for 10 minutes, then chased by the addition of excess cysteine and methionine. Glass-bead extracts corresponding to the indicated time points were prepared; immunoprecipitation was performed using anti-CPY antibodies, and the precipitates subjected to SDS-PAGE and fluorography. Positions of the p1, p2, and mature forms of CPY are indicated.

Figure 3

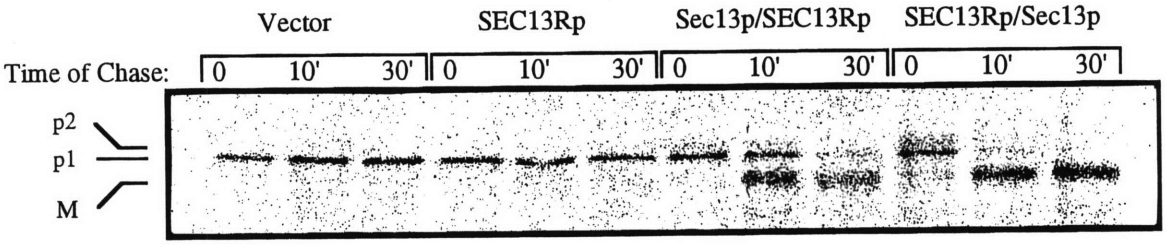

Vector
SEC13Rp
Sec13p/SEC13Rp
SEC13Rp/Sec13p
Time of Chase:
0
10'
30'
0
10'
30'
0
10'
30'
0
10'
30'
p2
p1
M

Figure 4

Complementation of a *SEC13*-deletion by the Sec13p/SEC13Rp chimera. 50 ug of protein extract from the indicated yeast strains was analyzed by western blotting using affinity-purified anti-Sec13p antibody, as described previously (Pryer et al., 1993). Lane 1, extracts from wild-type yeast; lane 2, from wild-type yeast expressing the Sec13p/SEC13Rp chimera; lane 3, from a *SEC13*-deletion strain expressing the Sec13p/SEC13Rp chimera.

Figure 4

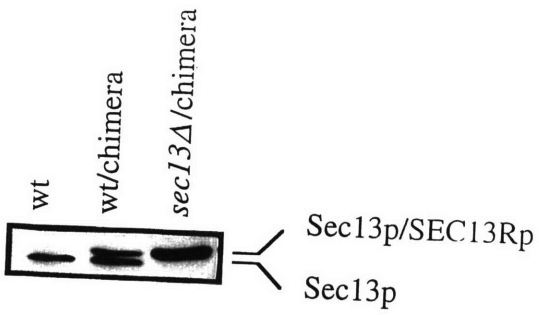


Figure 5

Specificity of SEC13Rp-induced growth inhibition. Indicated yeast strains containing a galactose-inducible SEC13Rp construct were incubated at 24°C in the presence of glucose or galactose. Strains used were: DSY174 (wild-type), DSY223 (*sec12*), DSY216 (*sec13*), DSY228 (*sec16*), DSY232 (*sec17*), DSY236 (*sec18*).

Figure 5

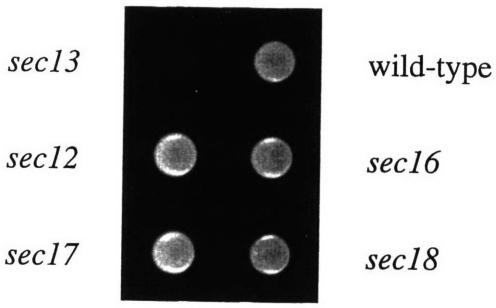


Figure 6

Specificity of antisera to the SEC13Rp protein. 50 ug of the indicated cell extracts were subjected to Western analysis and probed using the affinity-purified anti-SEC13Rp antibody. Lane 1, wild-type yeast; lane 2, wild-type yeast+pGAL-SEC13Rp; lane 3, CHO cells; lane 4, COS cells. The anti-SEC13Rp antibody recognizes a single predominant species of the predicted molecular weight, 36 kD.

Figure 6

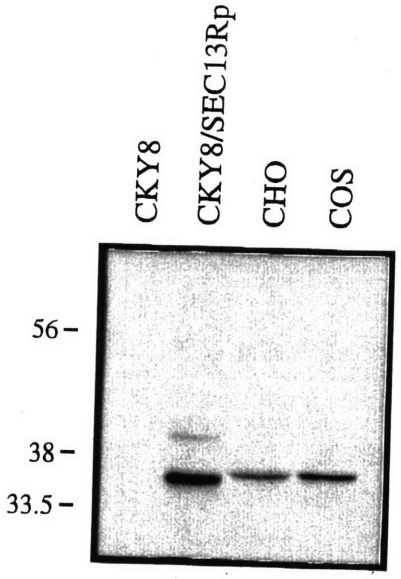


Figure 7

Localization of SEC13Rp by immunofluorescence. SEC13Rp appears in a perinuclear distribution in (a) CHO and (b) COS cells. Immunofluorescence performed using affinity-purified anti-SEC13Rp antibodies. Magnification: x700.

Figure 7

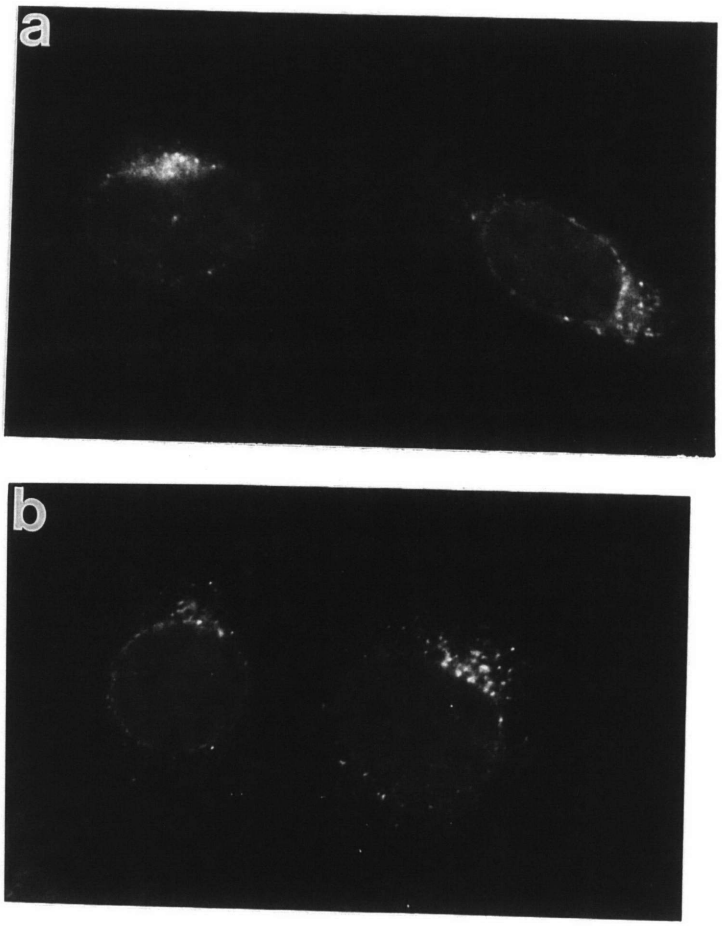


Figure 8

By electron microscope immunolabeling of (a) insulin or (b) acinar rat pancreatic cells, SEC13Rp is restricted to the transitional area of the ER. TE=transitional elements of the ER with associated transfer vesicles (asterisks); the arrow in the inset indicates a labeled bud on a transitional cisterna; G=Golgi complex; CV = condensing vacuole in the Golgi region. Note that the dense cytosolic matrix in the transitional area of Fig. 8b is also labeled in addition to transfer vesicles. See Table 1 for the quantitation of the immunogold labeling. Magnifications: a) x53,000 (inset x72,000); b) x54,000.

Figure 8

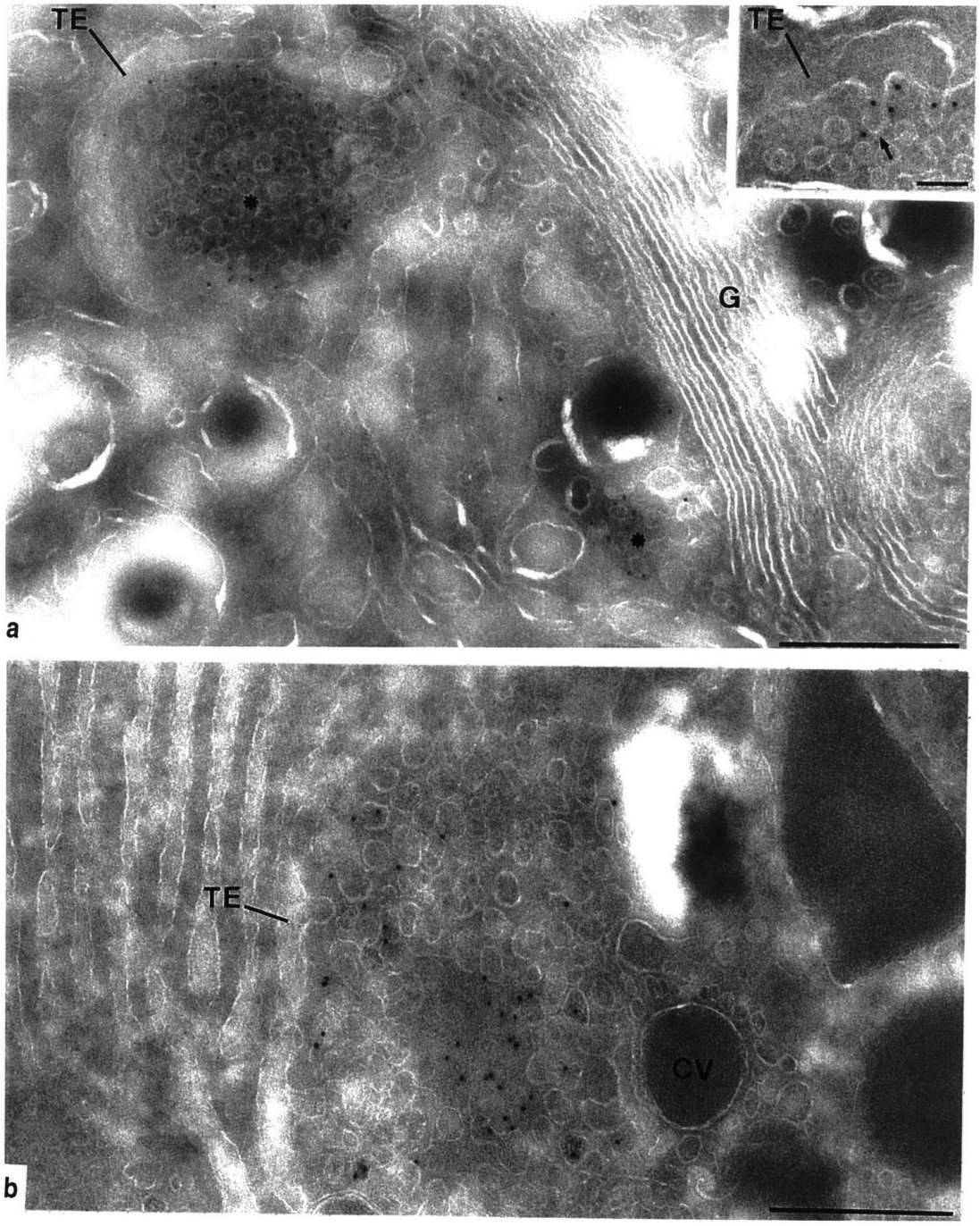
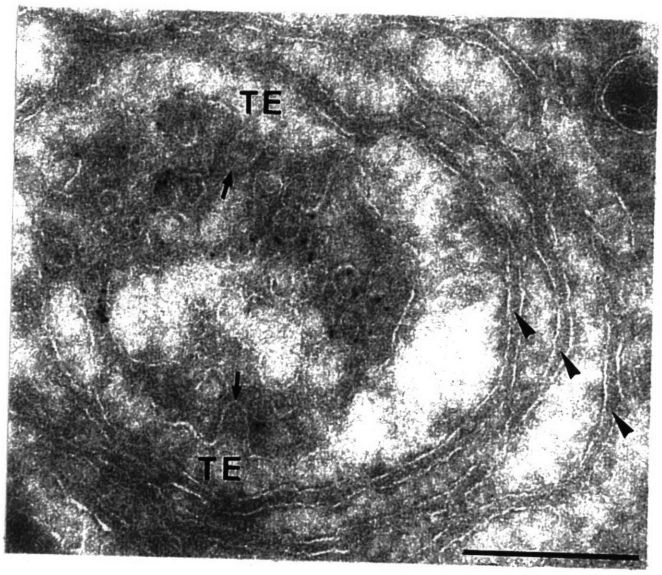


Figure 9

SEC13Rp labeling of a BFA body. Immunogold particles are present on the buds (arrows) and vesicles associated with the transitional endoplasmic reticulum cisterna (TE), but are absent from the dense bands of cytosol (arrowheads) enriched in coatomer (Orci et al., 1993a), situated between transitional and non-transitional ER cisternae. BFA-treated insulin cell. Magnification: x47,600.

Figure 9



Chapter III

Development of a two-hybrid approach for the identification of a mammalian homolog of the yeast Sec16p protein

ABSTRACT

The identification of mammalian homologs of the yeast COPII proteins Sec13p, Sec23p, and Sar1p suggests that the COPII coat assembly process has been evolutionarily conserved. Convergent evidence (reviewed in Chapter I) suggests that in yeast, Sec16p -- an essential protein tightly bound to the cytoplasmic face of the ER -- may also participate in the assembly of the COPII coat. This chapter describes the development and implementation of an approach to clone a mammalian homolog of Sec16p. This strategy was prompted by the observation that yeast Sec16p can interact with yeast Sec23p in a two-hybrid system. The cloning project involved three steps: first, a full-length mammalian Sec23 clone was isolated by hybridization using a partial Sec23 clone as a probe. Second, since the overexpression of both yeast and mammalian Sec23 was found to be toxic to cells, a two-hybrid vector was constructed that would allow for the inducible expression of LexA-fusion proteins; this allowed the screening a library of activation-domain fusion proteins capable of interacting with Sec23. Finally, an interaction-trap screen was performed; $5x10^6$ transformants were screened, and 59 positives were isolated and sequenced. None of the clones analyzed appear to represent a mammalian Sec16p homolog. Potential explanations for these results are discussed.

INTRODUCTION

In eukaryotic cells, the transport of proteins from the endoplasmic reticulum (ER) to the Golgi is mediated by membrane-bounded vesicles (Palade, 1975). In vitro analysis of vesicle formation in the yeast S. cerevisiae has demonstrated the requirement for three cytoplasmic factors: the Sec13p/Sec31p complex; the Sec23p/Sec24p complex; and the small molecular-weight GTP-binding protein, Sar1p (Salama et al., 1993; Barlowe et al., 1994). Convergent evidence also suggests that Sec16p may be involved intimately in the vesicle formation process. *SEC16*, an essential gene which exhibits synthetic lethal interactions with all five COPII genes, encodes a 240 kD protein tightly associated with the cytoplasmic face of the ER (Nakano et al., 1989; Kaiser and Schekman, 1990; Espenshade et al., 1995; Gimeno et al., 1995; Salama et al., 1997). Sec16p is required for ER to Golgi transport, and is found on ER-derived transport vesicles (Espenshade et al., 1995). In addition, Sec16p has been shown to bind Sec23p (Espenshade et al., 1995). (Since the work described in this chapter was performed, Sec16p has also been shown to bind Sec24p and Sec31p [Gimeno et al., 1996; Shaywitz et al., 1997]).

Mammalian homologs of three COPII genes have been characterized. A mammalian homolog of Sar1p was identified using degenerate PCR (Kuge et al., 1995). Two clones, Sar1a and Sar1b were isolated; these clones are 91% identical to each other at the amino acid level, and are 61-67% identical to Sar1p. Immunoelectronmicroscopy using antibodies raised against Sar1a (and also reactive with Sar1b) demonstrated that Sar1 is found on the cisternae of the transitional ER, on the proximal Golgi, and on the intervening vesicles. Inhibition of the ER to Golgi transport of the marker protein VSV-G in vivo

and in vitro using a deliberately designed dominant negative Sar1 mutant demonstrated the requirement for Sar1 in mammalian ER to Golgi transport.

A mammalian homolog of Sec13p, Sec13Rp, was discovered serendiptiously (Swaroop et al., 1994). Although *SEC13R* itself could not complement the temperature-sensitive *sec13-1* mutant, chimeric constructs representing the N-terminal half of either Sec13p or Sec13Rp, and the C-terminal half of the other protein, could both complement the *sec13-1* secretion defect (Shaywitz et al., 1995). Sec13p was also shown to localize to the cisternae and vesicles in the region of the transitional ER (Shaywitz et al., 1995). The localization of Sec13Rp was not affected by BFA treatment, in contrast to the localization of coatomer subunits; this insensitivity to BFA was shared by Sec23, and placed these two proteins in a different category from the proteins of the COPI complex. A recent study of Sec13Rp both confirmed these localization data and demonstrated that the addition of anti-Sec13Rp antibodies disrupted the ER to Golgi transport of the marker protein VSV-G in a semi-intact cell assay, further supporting the notion that the function of COPII has been evolutionarily conserved (Tang et al., 1997).

At the time the experiments in this chapter were performed, a murine cDNA sequence strongly homologous to the first 637 amino acids of the yeast Sec23p protein had been identified (Wadhawa et al., 1993). In addition, antibody raised against the yeast Sec23p protein appeared to cross-react with a mammalian Sec23p homolog, which was found to exhibit a subcellular localization pattern indistinguishable from that of Sar1 and Sec13 (Orci et al., 1991). More recently, two human Sec23 homologs have been identified, hSec23Ap and hSec23Bp (Paccaud et al., 1996). Antibodies were raised against the N-terminal region of hSec23A; these antibodies did not react with either hSec23B or Sec23p. The hSec23A protein complemented a yeast *sec23*

temperature sensitive mutant, and was localized, in mammalian cells, to the transitional ER region. Gel filtration analysis of HepG2 cytosol indicated that hSec23Ap (like yeast Sec23p) fractionated as a 350 kDa complex. Immunoprecipitation of hSec23A from clarified cytosol identified a prominent band with an apparent molecular weight of 110 kD; this is approximately the molecular weight of yeast Sec24p, the binding partner of Sec23p. Although a mammalian Sec24p homolog has not as yet been cloned, a cDNA encoding a protein with significant homolog to Sec24p has been reported (D38555; KIAA0079 gene product; Nomura et al., 1994).

Two-hybrid analysis using a constitutively-expressed LexA-Sec16p (amino acids 1645-2194) fusion and a galactose-induced activation domain-Sec23p fusion demonstrated that these two proteins could interact strongly (Espenshade et al., 1995). The association between Sec23p and the C-terminal region of Sec16p has also been observed in vitro (Gimeno et al., 1996). Attempts to perform the two-hybrid analysis using reciprocal constructs were unsuccessful; the constitutive overproduction of a LexA-Sec23p fusion was found to be toxic to the cell.

The strong association of Sec23p and Sec16p in a two-hybrid system, together with the availability of a mouse cDNA sequence representing a large region of Sec23, suggested an approach for the identification of a mammalian Sec16p homolog. First, the partial cDNA would be used to isolate a cDNA encoding the entire mammalian Sec23 gene. Then, a vector allowing the expression of Sec23 as an inducible LexA-fusion would be constructed; this was important because the available two-hybrid libraries were in activation-domain fusion vectors. Finally, a two-hybrid screen using the inducible LexA-Sec23 construct would be employed to isolate mammalian Sec16. This approach was also expected to isolate mammalian Sec24.

RESULTS

Inspection of the published sequence for a mammalian Sec23 homolog (Wadhawa et al., 1993) revealed the appearance of a frame-shift; in particular, the apparent loss of a thymine nucleotide at position 1699 of the mouse sequence disrupted the strong homology to Sec23p, homology which continued in a different frame. We elected to use this partial Sec23 sequence to probe a murine adipocyte library (generously provided by H. Lodish) in an effort to recover a full-length clone. 800,000 transformants were screened, and five positive clones were identified, representing two different full-length inserts. One of these clones was amplified by PCR to insert a convenient 5' restriction site. This PCR product was then cloned into the galactose-inducible expression vector pCD43, and complementation of the temperature-sensitive allele *sec23-1* (DSY245 [*MATa sec23-1 ura3-52 leu2,3-112 Gal+*]) was assessed over a range of galactose concentrations (Table 1); for comparison, a galactose-promoted yeast *SEC23* expression plasmid (pPE118) was also evaluated . Although DSY245 cannot grow at 30°C in the presence of vector, complementation was observed using the full-length mammalian Sec23 clone. This clone was also placed into the pEG202 LexA-fusion vector, to examine whether the constitutive expression of the full-length mammalian clone would be toxic. Although transformants grew extremely poorly, the LexA-Sec23p fusion protein was detectable in extracts prepared from these transformants by immunoblotting using anti-Sec23p antibody (data not shown). These results encouraged us to engineer a galactose-inducible LexA-fusion vector which would permit the regulated expression of the full-length

Sec23 protein. The construction of this plasmid, designated pGilda, is detailed in the "Experimental Methods" section of this chapter, below.

To identify human cDNA clones that interact with mammalian Sec23, a human brain cDNA library (kindly provided by D. Krainc and R. Brent) was transformed into DSY259, representing the EGY48 strain carrying the Sec23/pGilda plasmid pDS73 and the indicator plasmid pSH18-34. Since expression of Sec23 is toxic to cells, we could not identify interacting clones by a selection process, and were compelled instead to utilize a screen. $5x10^6$ transformants were obtained on 120 plates; this represents a density of approximately 42,000 colonies/plate. Following growth of colonies, transformants were replica plated to media containing galactose and Xgal. Blue colonies were identified on these plates after 1-2 days of growth. Generally, the blue colonies were not well-isolated, and sequential purification steps were required to obtain a single, positive clone. Yeast in the vicinity of the blue colony were selected with a toothpick and streaked on a glucose-containing plate, in effort to isolate single colonies; following growth of colonies, the plates were replica-plated to plates containing galactose and Xgal. This process was iterated until pure blue colonies were obtained.

The library plasmid was recovered from the purified yeast by performing a yeast mini-prep and transforming the DNA into the KC8 strain of bacteria cells (kindly provided by R. Brent). This strain contains a genetic lesion complemented by the *TRP1* gene on the library plasmid. Thus, by growing KC8 transformants on plates lacking tryptophan, bacteria containing the library plasmid were selected. Initially, the library clones were transformed into the strain RFY206 (Finley and Brent, 1994) and tested by mating assay against a panel of different LexA-fusion proteins, including Sed4p, Sec12p, Sec13p, Sec13Rp, four different Sec16p fragments, Iss1p (R.

Gimeno, 1996), as well as both yeast and mouse Sec23; the vectors pEG202 and pGilda were also included as negative controls. Approximately half of the clones interacting with mammalian Sec23 also interacted with yeast Sec23p; several clones interacted with Iss1p; other interactions were not detected. The clones interacting with Sec23p or Iss1p did not seem to define a useful subclass. For example, two clones which interacted with both Iss1p and yeast Sec23p were identified as S100B, a 91 amino acid calcium binding protein, and p68, an RNA helicase; these proteins bear no obvious homology to Sec16p or Sec24p. The balance of the library clones were retransformed into DSY257, and assayed for interaction with mammalian Sec23.

Of the 240 blue colonies identified, 186 were successfully isolated. Fifty of these were an identical false-positive (which produced a characteristic pattern upon the EcoRI/XhoI restriction digest used to determined the size of the library insert) that would activate transcription of the reporter gene even in the absence of the Sec23p plasmid; these were not considered further. 66 clones successfully retested by either mating assay or direct transformation into DSY257; preliminary sequencing was performed on 59 clones.

The results of this analysis are shown in Tables II and III. Of the 59 clones sequenced, 45 corresponded to either known genes or homologs of known genes, while 14 corresponded to expressed sequence tags (ESTs) in the database. None of the known genes identified are homologous to either Sec24p or Sec16p. In particular, although a mammalian homolog of Sec24p is present in the database (D38555; KIAA0079 gene product; Nomura et al., 1994), it was not identified in this screen.

The failure to identify overtly the Sec16p gene in the database was not surprising, since sequences corresponding to Sec16p had never been identified by BLAST. However, the expectation was that we might either identify a

novel sequence, corresponding to Sec16p, or else identify candidate EST's that might not appear to encode a Sec16p fragment as a result of a sequencing error. By aligning a candidate EST with both the library clone and with overlapping ESTs, we hoped to generate consensus sequences that might encode Sec16p. We applied this approach to the eight different ESTs identified by our screen. In each case, we were able to determine an in-frame protein sequence corresponding to both our library clone and to a collection of overlapping ESTs. Each of the protein sequences in Table III represents the consensus determined from 2-7 aligned sequences. These sequences were tested against the database (by BLAST) and were individually evaluated against the Sec16p protein sequence (by ALIGN). Extensive homology was not detected in any of the comparisons.

DISCUSSION

This paper describes our attempt to identify a mammalian homolog of the yeast Sec16p protein by a two-hybrid interaction screen. First, a full-length mammalian homolog of the yeast Sec23p was isolated by hybridization using as a probe a partial cDNA clone of the mammalian sequence. Second, a LexA-fusion vector, pGilda, was constructed to allow for the inducible expression of toxic proteins such as Sec23. Finally, a two-hybrid screen was performed using the full-length mammalian clone in the pGilda vector. Fifty-nine clones were sequenced, but a clone with extensive homology to Sec16p was not obtained. Potential reasons for this result will be considered systematically.

1. A mammalian homolog of Sec16p does not exist. This represents the null hypothesis, and remains in force until disproven. Nevertheless, the identification of mammalian homologs of Sec13p, Sec23p, Sec24p, and Sar1p strongly suggests that the process of COPII coat assembly has been evolutionarily conserved. Given the strong evidence that Sec16p is also involved in the process of COPII vesicle formation (reviewed in Chapter 1), it seems likely that a mammalian Sec16p exists.

2. Mammalian Sec16p exists but was not present in this library. This remains a possibility. Although the level of Sec16p mRNA expression has not been investigated, such a long transcript may be expressed at a low level, leading to underrepresentation of the clone in this cDNA library. It should be noted that the source of this library was human fetal brain, and all of the COPII proteins which have been examined to date are expressed in this organ (Shen et al., 1993; Swaroop et al., 1994; Paccaud et al., 1996).

3. The required region of mammalian Sec16p was not present in this library. The two-hybrid library used has an average insert size of 1.5 kb. Sec23p has been shown to interact with the extreme C-terminal region of Sec16p, and thus, it seemed reasonable to expect this region to be represented in this library, which was constructed by selection of poly (A)+ RNA followed by first-strand synthesis primed by oligo-dT. However, it is possible that the in-frame clone was not present in this library. It should be noted that our failure to identify mammalian Sec24 in this screen may result from the requirement for the N-terminal region of Sec24p for Sec23p interaction (Gimeno et al., 1996); thus, a library clone representing only the C-terminal region of Sec24p would not be expected to interact with the LexA-Sec23p protein.

4. The required region of mammalian Sec16p was present but failed to interact with mouse Sec23p. While it is possible that the murine Sec23p protein is unable to interact with human Sec16, this seems unlikely. Given the interactions demonstrated between mammalian Sec13 and yeast Sec31, as well as the cross-species reactivity seen for antibodies raised against human Sec23, human Sec13, and yeast Sec23, it seems reasonable to expect the mouse and human proteins to interact (Paccaud et al., 1996; Shaywitz et al., 1995; Orci et al., 1991).

5. Mammalian Sec16p was identified in this screen but we are not able to recognize its identity. This remains a concern. On the one hand, the mammalian homologs of Sec13p, Sec23p, Sec24p, and Sar1p are approximately 50% identical to the yeast protein at the amino acid level (Swaroop et al., 1994; Paccaud et al., 1996; Nomura et al., 1994; Kuge et al., 1995). Thus, if a similar homolog with mammalian Sec16 exists, we should easily be able to detect it. However, Sec16p appears to be an extremely

unusual protein, and in particular, it seems to consist of alternating proline-rich domains and highly-charged domains (Espenshade et al., 1995). It is possible that Sec16p could experience significant changes at the amino-acid level while still retaining its general function; for example, as pointed out by Gerhart and Kirschner (1997), structural similarity does not necessarily imply sequence similarity; while the amino-terminal 30 amino acid region of human and yeast histone H4 are identical between yeast and human, there are on average 13 differences in this sequence between *Tetrahymena* and human; nevertheless, a *Tetrahymena* protein differing by 25 amino acid substitutions can functionally replace the corresponding yeast protein in yeast, emphasizing that conservation of function does not necessarily require extensive conservation of amino acid sequence.

In summary, it is not possible to determine with precision exactly why a clone with homology to yeast Sec16p was not isolated in our screen. The identification of a mammalian Sec31p homolog (suggested by data presented in Chapter IV), together with the construction of a randomly-primed mammalian cDNA library, would offer another opportunity for the identification of a mammalian Sec16p homolog by a two-hybrid approach.

MATERIALS AND METHODS

General Techniques

Unless otherwise noted, yeast manipulations were performed as described (Kaiser et al., 1994). DNA manipulations were performed using standard techniques (Sambrook et al., 1989). PCR was performed using Vent polymerase according the manufacturer's specifications (NEB, Beverly, MA). DNA sequencing was performed using the Sequenase kit (United States Biochemical Corp., Cleveland, OH).

Library screening (identification of full-length murine Sec23 clone)

3T3-L1 cDNA library from differentiated day 8 adipocytes was generously provided by H. Lodish. Library colonies were screened by hybridization using the Genius digoxigenin-based system (Behringer), according to the manufacturer's instructions. Briefly, the partial mouse Sec23 cDNA clone (generously provided by M. Wadhawa) was labeled by random-primed labeling with digoxigenin-derivitivized-dUTP. Filter lifts (using Hybond-N nylon membranes [Amersham]) were performed on bacterial colonies, and DNA was immobilized to the filter by UV crosslinking following the alkaline lysis of the cells. Hybridization was performed at 65°C using 4 ng/ml of probe. The hybridized probes were immunodetected using alkaline-phosphatase-conjugated anti-digoxigenin antibody, and the colorometric substrates NBT (nitroblue tetrazolium) and X-phosphate (5-bromo-4-chloro-3-indolyl phosphate toluidium salt).

Two-hybrid assay

Potential protein-protein interactions were investigated using the two-hybrid protein interaction assay and the plasmids pEG202 (constitutive expression of LexA-fusion), pJG4-5 (galactose-regulated expression of activation domain-fusion), and pSH18-34 (lacZ reporter) (Gyuris et al., 1993). Interactions were evaluated in the strain EGY48 (Zervos et al., 1993); positive interactions were scored as blue colonies on SC medium (pH 7.0) containing 2% galactose and 40 mg/l X-gal.

Mating assays were performed by transforming the activation-domain fusion plasmids into EGY48, and transforming the LexA-fusion plasmids together with the indicator plasmid pSH18-34 into RFY206 (Finley and Brent, 1994). The strains were mated, and lacZ expression was tested by replica plating diploids on X-gal medium, as above. The following LexA-fusion plasmids were used in the mating assay: pRH108 (Sed4p); pRH102 (Sec12p); pRH157 (Sed13p); pAA35 (Sec13Rp); pPE59 (Sec16p [aa1-824]); pPE62p (Sec16 [447-1235]); pPE58 (Sec16p [1645-2194]); pPE74 (Sec16p [447-1737]); pPE249 (Iss1p) (R. Gimeno, 1996). The KC8 *E. coli* strain (*pyrF, leuB600, trpC, hisB463*) was kindly provided by R. Brent.

pGilda construction

The inducible LexA-fusion vector pGilda (named after the self-sacrificial Verdi heroine) was constructed as follows: first, the 0.7 kb BamHI/EcoRI fragment containing the bi-directional *GAL1/GAL10* promoters was subcloned from pCD43 (Shaywitz et al., 1995) into the polylinker of the *ARS/CEN, HIS3*-marked pRS313 vector (Sikorski and Heiter, 1989). This construct was then digested with EcoRI and XhoI; these sites were filled-in using klenow fragment, and the vector religated; this removed all the restriction sites in the polylinker between EcoR1 and XhoI

(inclusive). The resulting construct was then digested with BamHI and SacI, in preparation for the insert constructed as follows.

First, a PCR reaction was performed using pEG202 as the template, and the following primers: (a) sense: 5'-AACAATCGATATGAAAGCGTTAACG -3'. (b) antisense: 5' - AACAGAGCTCGGACGGATTACAACA -3'. Primer (a) inserts a ClaI site just prior to the LexA coding region, and primer (b) inserts a SacI site after the 3' region of the *ADH*-terminator sequence (basepairs 1745-1756). The product of this PCR was digested with ClaI and SacI. Next, the following two oligos were obtained and annealed: (1) 5' - GATCAAGGGTG -3'; (2) 5'- CGCACCCTT -3'. The 5' end of this adaptor is BamHI-compatible but corrupts the BamHI site; the 3' end is ClaI compatible, but corrupts the ClaI site. A three-way ligation was then performed, using the vector construct described above (digested with BamHI and SacI), the PCR fragment (digested with ClaI and SacI) and the adaptor (compatible with BamHI and ClaI). The resulting construct has been designated pGilda.

The full-length mammalian Sec23 construct was inserted into pGilda, to generate pDS73; the LexA-Sec23 fusion protein was specifically detected after galatose induction, and was not observed in extracts prepared from cells grown in glucose.

To demonstrate that pGilda could function in two-hybrid analysis, the gene encoding Sed4p (originally identified as a protein which interacts with Sec16p) was inserted into this vector, and tested against two different Sec16p activation-domain fusion constructs, pPE78 and pPE79. Sed4p specifically interacted with pPE79, and not with either pPE78 or with the vector alone. This same pattern of interaction was observed when Sed4p was expressed in pEG202; these data suggested that pGilda could substitute for pEG202 in the two-hybrid system.

Two-hybrid cDNA Library

cDNA library from human fetal brain was constructed in the activation domain fusion plasmid pJG4-5 (Gyuris et al., 1993) by D. Krainc (Mass. General Hospital), and represents 3.5 x 10^6 independent transformants. Average insert size is 1.5 kb.

TABLES

Table I: Complementation of *sec23-1* by mammalian *Sec23*

Temperature:	**36°C**				**30°C**			
% Galactose:	**0.002**	**0.02**	**0.2**	**2**	**0.002**	**0.02**	**0.2**	**2**
***GAL10-SEC23* (yeast)**	-	+	-	-	-	+	-	-
***GAL10-Sec23* (mouse)**	-	-	-	-	-	+	++	+
vector only	-	-	-	-	-	-	-	-

Growth assessed on synthetic complete medium lacking uracil and supplemented with 2% raffinose and the indicated concentration of galactose.

Table II: Known Genes Identified in Screen for Mammalian-Sec23 Interactors

Name	# clones	Remarks	% Proline	Accession #
Human heat-shock protein	6	DnaJ homolog	4.5	L08069
TFG	6	400 aa; present as Trk-fusion in thyroid cancer	12.2	Y07968
Threonyl tRNA synthetase	6		4.2	M63180
Pirin	4	nuclear protein	7.2	Y07867
S100B	3	91 aa calcium binding protein	0	P02638
Proteosome subunit C9	3		1.9	T60816
Translation Initiation Factor	1	similar to 4AII	2.7	X79538
p68	1	RNA helicase; DEAD-box protein; related to p72	5.0	P17844
p72	1	DEAD-box protein; related to p68	6.6	U59321
Desmoplakin	1	2677 aa; desmosome associated	1.9	M77830
Ribophorin	1	631 aa; oligosac.-prot. transferse subunit; ER loc.	4.0	Y00282
DAD-1	1	cell-death protein; subunit of olig.-prot. transferase	3.5	D15057
snRNP protein B	1		17.2	J04564
methylene-THF-reductase	1	cytoplasmic enzyme	5.8	J04031
TB2 (FAP locus)	1	gene deleted in familial adenomatous polyposis	4.1	M74090
Cyt. C oxidase subunit IV	1		3.6	X54802
KIAA0266	1	homologous to yeast YML093w ORF	6.0	D87455
hENT1	1	nucleoside transporter in pm	4.8	U81375
signal peptidase subunit	1	12 kD; canine gene in database	6.9	L38852
KIAA0069	1	unknown function	3.5	D31885
heat-shock protein	1	DnaJ homolog; different from above	5.1	Q04960
MeCP1	1	transcription factor	11.3	Y10746
Sec16p (yeast)			8.0	

Total number of clones represented: 45

Table III: Composite ESTs Identified in Screen for Mammalian-Sec23 Interactors

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AA305793 (4)
VYVKPGNKERGWNDPPQFSYGLQTQAGGPRRSLLTKRVAAPQDGSPRVPASETSPGPPPMG
PPPPSSKAPRSPPVGSGPASGVEPTSFPVESEARLMEDVLRPLEQALEDCRGHTRKQVCDDI
SR

R18915 (3)
AQAAVQGPVGTDFKPLNSTPATTTEPPKPTFPAYTQSTASTTSTTNSTAAKPAASITSKPATLT
TTSATSKLIH

AA043465 (2)
VKNMSSLEISSSCFSLETKLPLSPPLVEDSAFEPSRKDMDEVEEKSKDVINFTAEKLSVDEVSQL
VISPLCGAISLFVGTTRNNFEGEKSH*

AA295402 (2)
PPGAPPFLRPPGMPGLRGPLPRLLPPGPPPGRPPGPPPGPPPGLPPGPPPRGPPPRLPPPAP
PGIPPTRPGMMRPPLVPPLGPAP

AA313126 (1)
RDHRRYFYVNEQSGESQWEFPDGEEEEEESQAQENRDETLAKQTLKDKTGTDSNSTESSETS
TGSLCKESFSGQVSSSSLMPLTPFWTLL

H90481 (1)
NRGSEVIAAGMVVNDWCAFCGLDTTSTELSVVESVFKLNEAQPSTIATSMRDSLIDSLT*

AA315015 (1)
TNNTPMNQSVPRYPNAVGFPSNSGQGLMHQQPIHPSGSLNQMNTQTMHPSQPQGTYASPPP
MS

N49984 (1)
GACTSRPIHPSKAPNYPTEGNHRVEFNVNYTQDLDKVMSGSERN

Composite ESTs assembled from 2-7 overlapping sequences;
Accession number of representative EST is indicated;
Number of library clones corresponding to each composite sequence is indicated;
Total number of clones represented: 15
"*": STOP codon

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Chapter IV

COPII subunit interactions in the assembly of the vesicle coat

PREFACE

This chapter has been submitted for publication as: David A. Shaywitz, Peter J. Espenshade, Ruth E. Gimeno, and Chris A. Kaiser (1997): COPII subunit interactions in the assembly of the vesicle coat.

Peter Espenshade provided the recombinant Sec23p and Sec24p, and was instrumental in the development of the in vitro binding assays used in this study. Ruth Gimeno contributed to informative pilot experiments that first suggested an interaction between Sec24p and Sec31p.

Summary

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

Introduction

In eukaryotic cells, proteins which enter the secretory pathway are synthesized in the endoplasmic reticulum (ER)[1], then transported to the Golgi apparatus via membrane-bounded vesicles (1). Vesicles are formed by the recruitment and assembly of cytosolic coat components upon the donor ER membrane (2, 3). Dissection of vesicle formation in the yeast *S. cerevisiae* has been facilitated by both the discovery of conditional mutants that are defective in vesicle formation, and by in vitro reconstitution studies which have helped define the essential components of the vesicle assembly machine. In particular, three cytoplasmic factors, collectively termed COPII, have been shown to be required for vesicle formation: the Sec31p/Sec13p protein complex, the Sec23p/Sec24p protein complex, and the small GTP-binding protein Sar1p (4, 5). The addition of these three factors to urea-washed ER membranes stimulates the formation of coated, fusion-competent vesicles (4).

While in vitro analysis has been able to define soluble components required for vesicle assembly, membrane-associated factors have remained less accessible to biochemical study. Thus, the mechanism of COPII recruitment and assembly onto the membrane is still unknown. However, converging genetic and biochemical evidence suggests that Sec16p, an essential 240 kD multidomain protein, may be involved intimately in this process. *SEC16* is required for transport vesicle formation (6, 7), and exhibits genetic interactions with all five COPII genes (7-10). Sec16p is tightly associated with the periphery of the ER, and is also found on ER-derived transport vesicles (6); furthermore, Sec16p cannot be extracted from membranes by urea (6). Sec16p has been shown to physically associate with the COPII subunits Sec23p and Sec24p (6, 11). In addition, membranes

prepared from *sec16* mutant strains exhibit a marked deficit in the release of vesicle cargo molecules when assayed in vitro (12). Finally, the addition of antibody against Sec16p to the in vitro budding reaction inhibits the formation of COPII vesicles.[2]

To define more precisely the role of Sec16p, we employed a yeast two-hybrid interaction screen. We found that the COPII protein Sec31p interacts with Sec16p, Sec23p, and Sec24p, as well as showing an expected interaction with Sec13p. These results were confirmed by in vitro binding studies using full-length Sec31p or truncations of Sec31p, expressed and purified from yeast extracts as glutathione S-transferase (GST) fusion proteins. The in vivo significance of the binding of Sec31p to Sec16p was explored using a Sec31p truncation mutant specifically defective for Sec16p binding. This mutant could not complement a *sec31*-null mutant nor rescue the secretion defect of the temperature-sensitive *sec31-2* mutant at non-permissive temperatures. These findings support the hypothesis that Sec16p functions as a foundation for the assembly of the COPII coat on the ER membrane.

Experimental Procedures

General Techniques -- Yeast manipulations were performed by standard methods (13). Western blotting was performed using the following antibodies: anti-HA (12CA5; 1/1000; BAbCO); anti-Sec23 (1/250) (14); anti-Sec24p (1/250) (14); anti-Sec13p (1/250) (15); anti-Sec31p (1/10,000) (5). The antibodies against Sec23p, Sec24p, and Sec31p were generously provided by R. Schekman.

Two-hybrid Analysis -- The two-hybrid screen was performed in the indicator strain L40 (16); subsequent analysis utilized CKY556, representing the strain EGY40 (17) transformed with the indicator plasmid pSH18-24 (18). The L40 strain was initially transformed with the LexA-Sec16p fusion plasmid, pDS99, representing the coding sequence for Sec16p amino acids 447-1235 inserted into the pBTM116 vector (19); this new strain, designed CKY554, was then transformed with an activation-domain fusion library (generously provided by M. White) in the pGADGH vector (20). Interactions were tested as described (18, 21). Sec31p truncation constructs in pGADGH were subsequently constructed using the cloned *SEC31* locus (10), generously provided by R. Schekman. LexA-fusion constructs were made in pBTM116, and represent the entire coding sequence of yeast Sec13p (pDS138) (15) and human Sec13Rp (pDS168) (22, 23), and amino acids 666-926 of Sec24p (pDS272) (8) and amino acids 447-1043 of Sec16p (pDS116) (6). The entire coding sequence of yeast Sec23p (14) was inserted into pGilda, to generate pDS72. pGilda represents a derivative of the pEG202 *lexA* fusion vector (18) that retains the multiple cloning sites of pEG202 but which utilizes the *GAL1* promoter instead of the *ADH1* promoter; the vector backbone of pGilda is from pRS313 (24). To assay quantitatively production of the *lacZ* reporter gene, the CKY556 strain was co-transformed with LexA- and activation

domain-fusion plasmids. Transformants containing a pBTM116-derived LexA-fusion plasmid were grown to exponential phase in selective medium containing glucose. Transformants containing a pGilda-derived LexA-fusion plasmid were grown to exponential phase in selective medium containing 2% raffinose; then galactose was added to 2%, and growth continued for another 4 hours. At least three independent transformants were assayed for β-galactosidase activity (13). The mean activity of the transformants is given, and expressed in Miller units (1000 x OD_{420}/[reaction time x OD_{600} units assayed]) (25). All values above background were within 35% of the mean value.

In vitro Binding Studies -- Recombinant Sec23p and Sec24p were prepared as previously described (11). Recombinant Sec13p (26) was generously provided by K. Saxena and E. Neer. The Sec16 protein utilized in these experiment was prepared as follows: a DNA fragment encoding amino acids 447-1043 of Sec16p was cloned into the *GAL10*-promoted HA expression vector pRH165 (R. Gimeno, unpublished), to generate pDS216. This plasmid was transformed into the *S. cerevisiae* strain CKY557 (*MATα ura3-52 trp1::hisG GAL*$^+$). Cells were grown to exponential phase in selective medium containing 2% raffinose, then supplemented with 2% galactose for 4-6 hours to induce expression of the epitope-tagged protein. These cells were then washed in LBB-100 (20 mM HEPES-KOH, pH 6.8, 80 mM KOAc, 5 mM MgOAc, 0.02% Triton X-100, 0.1M NaCl) supplemented with protease inhibitors PMSF (1 mM), leupeptin (0.5 ug/ml), pepstatin (0.7 ug/ml) as well as EDTA (0.5 mM), then frozen by drops in liquid nitrogen. Frozen cell pellets were lysed using a mortar and pestle and resuspended in LBB-100. The lysate was cleared by centriguation at 3,000 x g for 5 min, followed by centrifugation at 100,000 x g for 40 min.

DNA regions encoding the entire Sec31p protein, a fragment of Sec31p lacking the initial 490 amino acids, or a fragment of Sec31p lacking the final 98 amino acids were fused to the 3' end of the glutathione S-transferase (GST) coding sequence under the control of the *GAL1* promoter in the expression vector pPE127, a vector identical to pRD56 but in a different reading frame (6). Clarified cytosolic extracts were prepared as described above, except that the concentration of NaCl used was 0.6 M; this buffer is referred to as LBB-600. Glutathione-Sepharose 4B beads (Pharmacia) were incubated with the extracts for 30 min at 25°C, then washed three times with LBB-600. Beads prepared in this fashion were decorated with 2-5 pmol of the fusion protein. For the binding reactions involving Sec23p, Sec24p, and Sec13p, the decorated beads were washed twice with binding buffer (25 mM K-Hepes pH 6.8, 0.1% Triton X-100, 1 mM MgCl2, 0.25 mg/ml BSA). The beads were then resuspended in 45 µl of salt-supplemented binding buffer, and 5 µl of the relevant recombinant protein was then added. Binding of Sec23p (2 pmol) and Sec24p (1 pmol) was carried out in 50 mM NaCl, while binding of Sec13p (3 pmol) was carried out in 150 mM NaCl. After incubation for 1 hour at 25°C, the beads were washed 2-3 times with binding buffer (no additional salt), then resuspended in extract sample buffer (ESB; ref. 11). For the reactions involving Sec16p, the beads were washed twice with LBB-100, then incubated with a yeast cytosolic extract prepared as described. These binding reactions were carried out in a volume of 100 µl, and utilized extract containing 126 µg total protein; NaCl was added to a final concentration of 0.2 M. Following a one hour incubation at 25°C, the beads were washed twice with LBB-100, then resuspended in ESB. All proteins were subjected to SDS-PAGE followed by Western blot analysis.

In vivo Complementation Studies -- Both the *SEC31*-deletion strain RSY1109 (*MAT* ***a*** *ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-Δ1 sec31::TRP1* [pNS3111-*SEC31-URA3-CEN*]), kindly provided by R. Schekman, and the temperature-sensitive *sec31-2* strain CKY555 (*MAT****a*** *sec31-2 ura3-52 leu2-3,112*), kindly provided by A. Frand, were transformed with pDS321, pDS327, pDS328, or pRS415 (Stratagene). *sec31-2* was identified by A. Frand in a screen for new mutants temperature-sensitive for ER to Golgi transport; the mutation in *sec31-2* was mapped by marker-rescue of gapped plasmids to a region corresponding to amino acids 850-1175 (data not shown). pDS321 contains the full *SEC31* genomic locus (6.2 kb *BamHI/PstI* fragment) inserted into the *CEN, LEU2*-marked pRS415 vector. pDS327 and pDS328 both contain a 5.0 kb *SalI-SalI* genomic *SEC31* fragment which represents a truncation that removes the coding sequence for the C-terminal 98 amino acids of the protein. pDS327 is a *CEN*-based plasmid, derived from pRS415, and pDS328 is a 2μ-based plasmid, derived from pRS425 (27). For the pulse-chase analysis, strains were grown to exponential phase at permissive temperature (24°C), then shifted to non-permissive temperature (36°C) for 20 min. Pulse-labeling of cells and immunoprecipitation of CPY were performed as previously described (23).

Results and Discussion

Portions of the Sec16p coding sequence were surveyed for regions that would not by themselves act as transciptional activators when fused to a DNA-binding domain, and would therefore be suitable for two-hybrid analysis. pDS99, representing the coding sequence for amino acids 447-1235 of Sec16p inserted into the pBTM116 LexA-fusion vector (19), was one of the constructs that fulfilled this criterion. This fragment of Sec16p includes the region known to bind Sec24p (Sec16p amino acids 565-1235), and is distinct from the region known to bind Sec23p (Sec16p amino acids 1638-2194) (6). The L40 reporter strain (16) was transformed with both pDS99 and a *S. cerevisiae* cDNA library constructed in the activation domain-fusion vector pGADGH (20). Library plasmids were recovered from strains positive for expression of both the *HIS3* and *lacZ* reporter genes. A screen of $8x10^5$ *S. cerevisiae* cDNA clones yielded seven positives whose activation of *lacZ* reporter expression depended upon the presence of the LexA-Sec16p fusion protein. Six of the positive clones contained overlapping cDNA segments derived from the 3' region of the *SEC31* gene (10). The smallest of these clones, 2a8, encodes a peptide of 127 amino acids, representing the extreme C-terminus of the 1273 amino-acid Sec31p molecule.

In an effort to define the functional domains of Sec31p, a series of Sec31p deletions were constructed in pGADGH and evaluated by two-hybrid analysis against a series of potential interactors constitutively expressed as LexA-fusion proteins in the pBTM116 vector (Fig. 1). From this study, the Sec13p-binding region of Sec31p mapped to the N-terminal third of the Sec31p protein, a region which contains six WD-40 repeats (28, 29). Constructs expressing at least the first 490 amino acids of Sec31p, such as pDS131 (Fig. 1), interacted strongly with Sec13p, while constructs lacking this region failed to

interact. Since Sec13p itself consists almost entirely of WD-40 repeats (26), the interaction of Sec13p with the N-terminal region of Sec31p indicates that these regions of WD-40 repeats associate in a homotypic fashion.

We also examined the interactions of the human Sec13p homolog, Sec13Rp (22, 23). Sec13Rp can function in yeast (23); in mammalian cells, it is required for ER to Golgi transport (30), and has been localized to the cisternae and vesicles in the region of the transitional ER (23, 30). Human Sec13Rp exhibited a two-hybrid interaction profile identical to that of yeast Sec31p, interacting specifically with pDS131. These results emphasize the degree of conservation between yeast and mammalian COPII structures (23, 30-32), and strongly imply the existence of a mammalian Sec31 protein.

We next asked whether Sec31p could interact with either Sec23p or Sec24p. Utilizing a domain of Sec24p that does not interact with Sec23p (11), we determined that Sec24p interacts with the central region of Sec31p (pDS134), a region that does not interact with either Sec13p or Sec16p. The evaluation of Sec23p binding to Sec31p required the use of an inducible LexA-Sec23p fusion protein, since the constitutive overexpression of fusions to *SEC23* fusion was lethal (data not shown). We constructed the vector pGilda, which allows for the galactose-inducible expression of toxic LexA-Sec23 fusion proteins, and found that Sec23p interacted specifically with a central, 325-amino acid region of Sec31p (pDS135). Since the analysis of pGilda-expressed fusion proteins requires different growth conditions than the analysis of pBTM116-expressed fusion proteins, the ß-galactosidase activity observed in strains transformed with the different plasmids, while internally consistent, are not directly comparable. Both the background ß-galactosidase activity and the ß-galactosidase activity in strains containing interacting plasmids were slightly higher in strains carrying pGilda-derived plasmids.

To confirm the interactions detected by two-hybrid analysis, we asked whether Sec16p, Sec23p, Sec24p, and Sec13p could bind to different GST-Sec31p fusion proteins isolated from yeast. This approach has been used previously to demonstrate the direct binding of Sec23 and Sec24p to different regions of Sec16p (11). We evaluated three different GST-fusion proteins: full-length Sec31p, Sec31p lacking the N-terminal 490 amino acids (Sec31ΔNp), and Sec31p lacking the C-terminal 98 amino acids (Sec31ΔCp). Sec23p and Sec24p were expressed as GST-fusion proteins in *E. coli* , purified by affinity chromotography, then released by thrombin cleavage of the GST moiety (11). Sec13p (kindly provided by K. Saxena and E. Neer) was purifed as a hexa-His-fusion protein from *E. coli* (26). Clarified extracts of yeast overexpressing the central-domain of Sec16p (amino acids 447-1043) which had been tagged with a hemagglutinin (HA) eptitope provided the source of Sec16p for this experiment. This fragment contains the putative Sec31p-interacting region but is more soluble than the full-length protein.

The results from these binding experiments (Fig. 2) were in complete agreement with the two-hybrid data. Full-length Sec31p was able to bind Sec13p, Sec23p, Sec24p, and Sec16p. However, Sec31ΔNp was specifically defective for Sec13 binding, while Sec31ΔCp was specifically defective for Sec16 binding. Sec23p and Sec24p bound to both of the truncated proteins, but not to GST alone.

The identification of Sec31ΔCp, which is specifically defective for Sec16p binding, allowed us to investigate the in vivo significance of this interaction (Fig. 3). First, we asked whether *sec31-ΔC* could functionally substitute for wild-type *SEC31*. A *sec31*-null strain bearing wild-type *SEC31* on a *URA3*-marked plasmid was transformed with *LEU2*-marked plasmids carrying either *SEC31* or *sec31-ΔC*. Transformants were grown with selection

for the *LEU2*-marked plasmid and then plated on medium containing 5-fluoroorotic acid (5-FOA). Only yeast capable of growing in the absence of the *URA3*-marked plasmid would be expected to grow under these conditions. The strains carrying the plasmid with *sec31-ΔC* did not produce segregants that could grow on 5-FOA (Fig. 3A), showing that the truncated protein lacks an essential function of Sec31p.

As a control for protein expression levels, the wild-type yeast strain CKY8 (6) was transformed with either a *CEN*-plasmid carrying *sec31-ΔC* or with vector alone. Extracts from both strains were examined by immunoblotting using anti-Sec31p antibody (Fig. 3D) (5). Sec31ΔCp was present in equivalent amounts to the endogenous Sec31p, indicating significant production of the truncated protein.

To address more directly the role of the C-terminal region of Sec31p in secretion, we utilized a temperature-sensitive allele of *SEC31*, designated *sec31-2*, which was isolated in a screen for new mutants defective for ER to Golgi transport. By testing whether Sec31ΔCp could rescue the secretion defect of *sec31-2* observed at non-permissive temperatures, the ability of Sec31ΔCp to fulfill the function of Sec31p in ER to Golgi transport could be assessed. The *sec31-2* mutant was transformed with plasmids encoding either Sec31p or Sec31ΔCp; growth of the transformants at the non-permissive temperature of 36°C was then evaluated. While mutants transformed with the *SEC31* plasmid grew at 36°C, mutants transformed with the *sec31-ΔC* plasmid remained temperature-sensitive for growth (Fig. 3B); these results were observed in mutants transformed with either a *CEN*-based or a 2μ-based *sec31-ΔC* plasmid. The kinetics of ER to Golgi transport of the marker cargo protein carboxypeptidase Y (CPY) was followed by pulse-chase analysis of the transformants at 36°C. The *sec31-ΔC* plasmid did not rescue the CPY transport

defect (Fig. 3D). Because the binding studies showed that the only apparent defect of Sec31ΔCp is in binding to Sec16p, the transport defect exhibited by the truncated allele of *SEC31* argues that the binding of Sec31p to Sec16p is required for ER to Golgi transport. However, we cannot eliminate the possibility that the C-terminal region of Sec31p performs an additional function which has not yet been defined that is necessary for secretion.

Reconstitution studies in both yeast and mammalian cells demonstrate that vesicle coat formation can be stimulated by the addition of a defined set of cytosolic factors to washed donor membranes (4, 5, 33). For transport between Golgi cisternae in mammalian cells, these factors are the small GTP-binding protein ARF and the coatomer protein complex, consisting of seven subunits which coassemble in the cytosol and bind *en bloc* to the donor membrane (34). For transport between yeast ER and Golgi, two different cytosolic protein complexes in addition to the small GTP-binding protein Sar1p are needed to form the COPII vesicle coat (3, 14,15). The interaction that we have detected between purified components of the Sec31p/Sec13p protein complex and the Sec23p/Sec24p protein complex suggested that these two complexes may pre-assemble in the cytosol. To examine this possibility, we expressed a GST-Sec31p fusion protein in yeast and asked whether Sec23p or Sec24p could could be found associated with this fusion protein in a cytosolic extract prepared under conditions of the in vitro transport assay (4, 15). We were unable to detect either of these proteins in the bound fraction. This observation is consistent with our measurements of the stability of the interactions between isolated proteins: binding of GST-Sec31p to recombinant Sec23p and Sec24p was detected at 50 mM NaCl, but was not seen at 150 mM NaCl, a salt concentration equivalent to that used for the in vitro assay (data not shown). Under the same conditions, binding of GST-Sec31p to Sec13p or

Sec16p was stable (Fig. 2). Consequently, it is likely that the physiological association between the two COPII complexes requires the context of the ER membrane.

Conditional mutants of *SEC16* exhibit synthetic lethal interactions with conditional mutants of *SEC13*, *SEC23* (7), *SAR1* (8), *SEC31* (10), and *SEC24*[3]. Given that *SEC16* interacts genetically with all five COPII genes and encodes a peripheral ER membrane protein that is present on ER-derived transport vesicles, required for vesicle formation, and binds directly to Sec23p, Sec24p, and Sec31p, we propose that Sec16p functions as a foundation for the construction of the COPII coat from soluble protein complexes (Fig. 4). The demonstration that Sec31p binds directly to both Sec23p and Sec24p suggests that the assembling COPII subunits are stabilized not only by interactions with Sec16p but also by interactions with each other. Progress in understanding the stepwise mechanism of COPII recruitment and assembly may come from more precise definition of how the individual protein-protein interactions between COPII subunits that we describe here are controlled.

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Footnotes:

1. Abbreviations: ER, endoplasmic reticulum; GST, glutathione S-transferase; HA, hemagglutinin. PAGE, polyacrylamide gel electrophoresis. CPY, carboxypeptidase Y; 5-FOA, 5-fluoroorotic acid

2. P. Espenshade and C. Kaiser, unpublished results.

3. A. Frand, R. Gimeno, and C. Kaiser, unpublished results.

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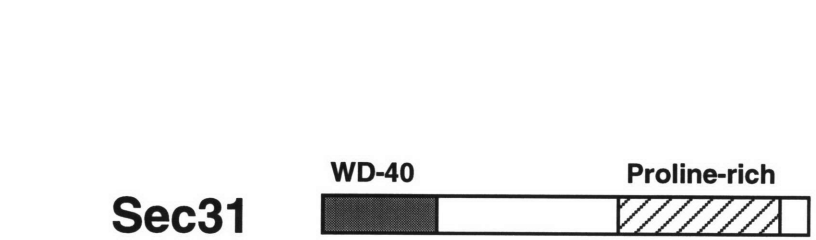
Figures

Figure 1.

Dissection of Sec31p binding domains through the use of two-hybrid analysis. The clone 2a8, a cDNA expressed in the activation-domain fusion pGADGH and encoding the C-terminal 127 amino acids of Sec31p, was identified in a two-hybrid screen for proteins that interact with the central region of Sec16p (amino acids 447-1235), expressed from the LexA-Sec16p fusion plasmid pBTM116. To define more precisely the domain structure of Sec31p, a series of Sec31p truncations were constructed in pGADGH. The peptide fragments represented by the activation-domain fusions are: pDS131 (1-490); pDS134 (570-1175); pDS135 (850-1175). These plasmids were evaluated against a series of *LexA*-fusions to *SEC13*, *SEC24* (coding sequence for amino acids 666-926), *SEC23*, and *SEC16* (coding sequence for amino acids 447-1043) for expression of a *lacZ* reporter. Analysis of Sec23p interactions required the inducible expression of the LexA-Sec23p fusion protein with *GAL1*-regulated vector pGilda. The regions of Sec31p that contain WD-40 repeats and proline-rich sequences are indicated in the diagram.

Figure 1



	LexA fusion: pBTM116				pGilda
	Sec13	Sec13R (human)	Sec24	Sec16	Sec23
pDS131	**217**	**48**	<1	<1	5
pDS134	<1	<1	**27**	<1	**462**
pDS135	<1	<1	<1	<1	**272**
2a8	<1	<1	<1	**31**	3

β-galactosidase activity (Miller Units)

Figure 2.

Binding of COPII subunits to Sec31p in vitro. (**A**) GST-fusion proteins representing full-length Sec31 (1273 amino acids), a N-terminal truncation lacking the first 490 amino acids (Sec31ΔN), a C-terminal truncation lacking the final 98 amino acids (Sec31ΔC), or GST alone were immobilized on gluthathione agarose and incubated with recombinant Sec23p, Sec24p, Sec13p, or clarified extract from yeast overexpressing the central domain of Sec16p (amino acids 447-1043) tagged with a hemagglutinin (HA) epitope. The bound fraction was resolved by SDS-PAGE, and proteins were detected by Western blotting. A sample representing the total amount of protein added to the reaction was also included for each protein analyzed; ratio of total to bound loaded is 1:1 for the three recombinant proteins, and 1:10 for the Sec16p extract. The recombinant Sec13p migrates slightly slower than endogenous Sec13p, and the doublet in the Sec13p lanes indicates that both proteins co-purify with Sec31p and Sec31ΔCp. (**B**) The affinity-purified GST-Sec31 fusion proteins used in the binding reactions were separated by SDS-PAGE on a 10% gel and stained with Coomassie brilliant blue.

Figure 2a

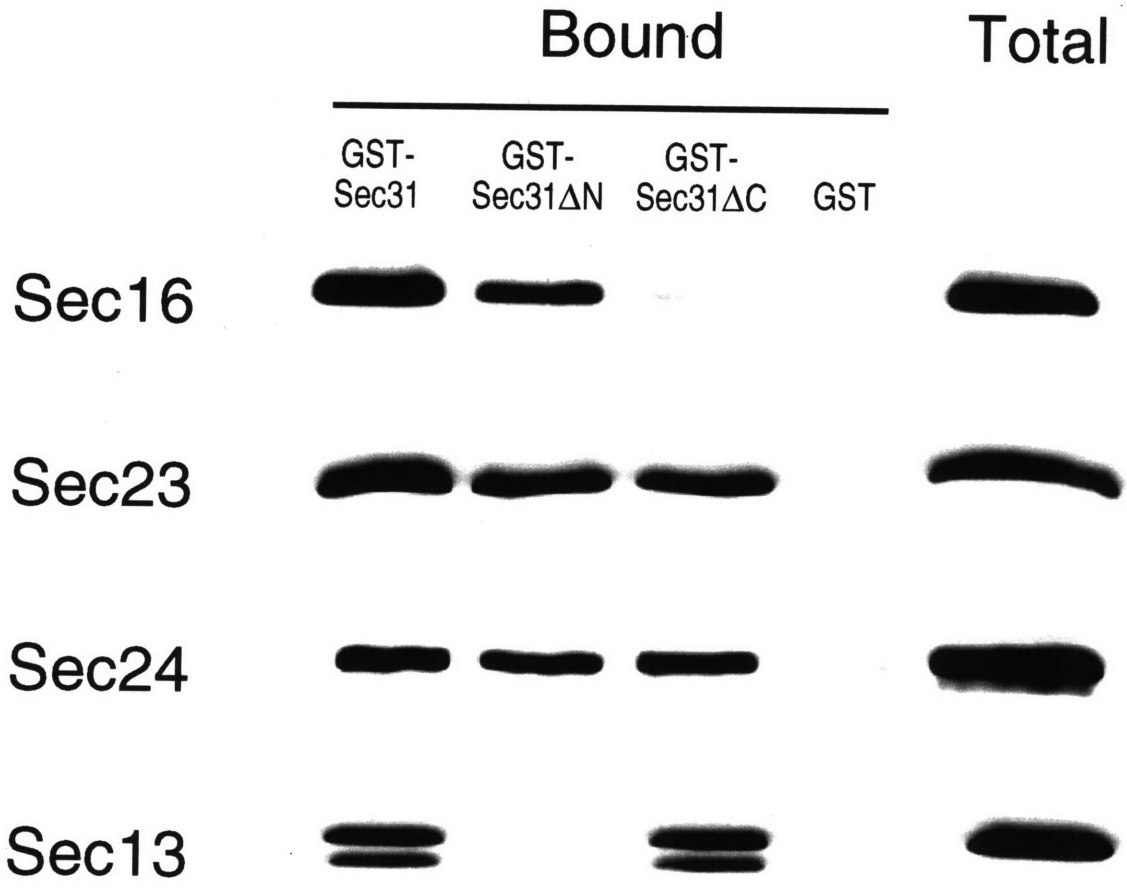

Bound
Total
GST-Sec31
GST-Sec31ΔN
GST-Sec31ΔC
GST
Sec16
Sec23
Sec24
Sec13

Figure 2b

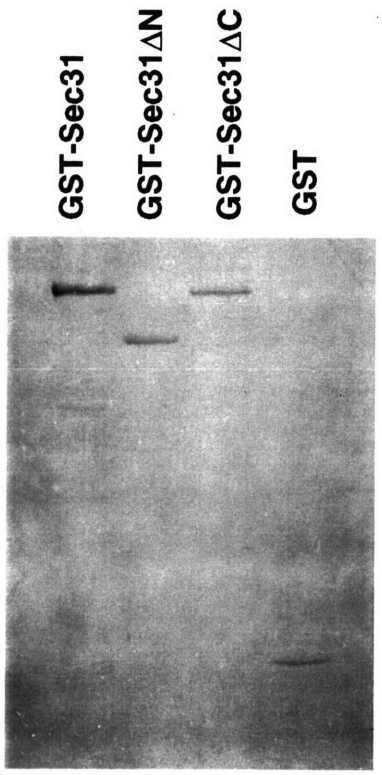


Figure 3

Sec16p-binding region of Sec31p is essential and required for ER to Golgi transport. Plasmids expressing either Sec31p (*CEN*- plasmid) or Sec31pΔC (both *CEN*- and 2μ-plasmids) were transformed along with a vector-only control into either RSY1109, which carries a chromosomal deletion of the entire coding sequence of *SEC31* covered by *SEC31* on a *URA3*-containing plasmid, or CKY555, which carries the temperature-sensitive allele *sec31-2*. (**A**) RSY1109 transformants were plated on medium containing 0.1% 5-fluoroorotic acid (5-FOA) to assay for complentation of the null allele. (**B**) CKY555 transformants were plated at both permissive (24°C) and non-permissive (36°C) temperatures, to assay complementation of the *sec31-2* allele. (**C**) The transport of the vacuolar protein carboxypeptidase Y (CPY) in the CKY555 transformants at non-permissive temperatures was examined by pulse-chase analysis. Exit of CPY from the ER is observed only in the strain expressing the full-length wild-type Sec31p protein. Positions of the p1 (ER), p2 (Golgi), and mature (vacuole) forms of CPY are indicated, as is the time in minutes of chase. (**D**) Expression of Sec31ΔCp. Extracts were prepared from the wild-type yeast strain CKY8 (6) transformed with either *sec31-ΔC* on a *CEN*-plasmid (lane 1) or vector only (lane 2); samples were resolved by SDS-PAGE and transfered to nitrocellulose. Proteins were detected by Western blotting using anti-Sec31p antibodies.

Figure 3

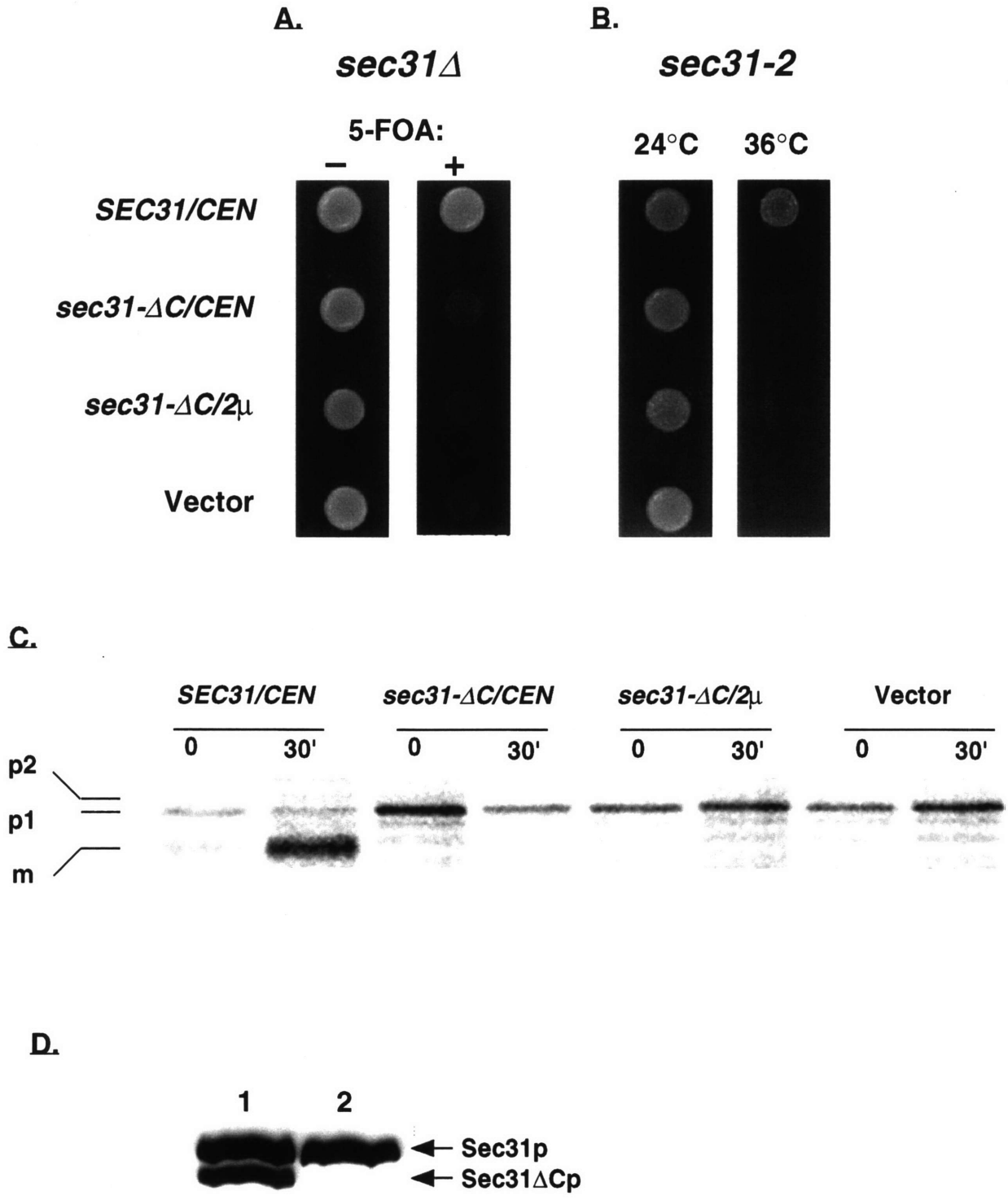
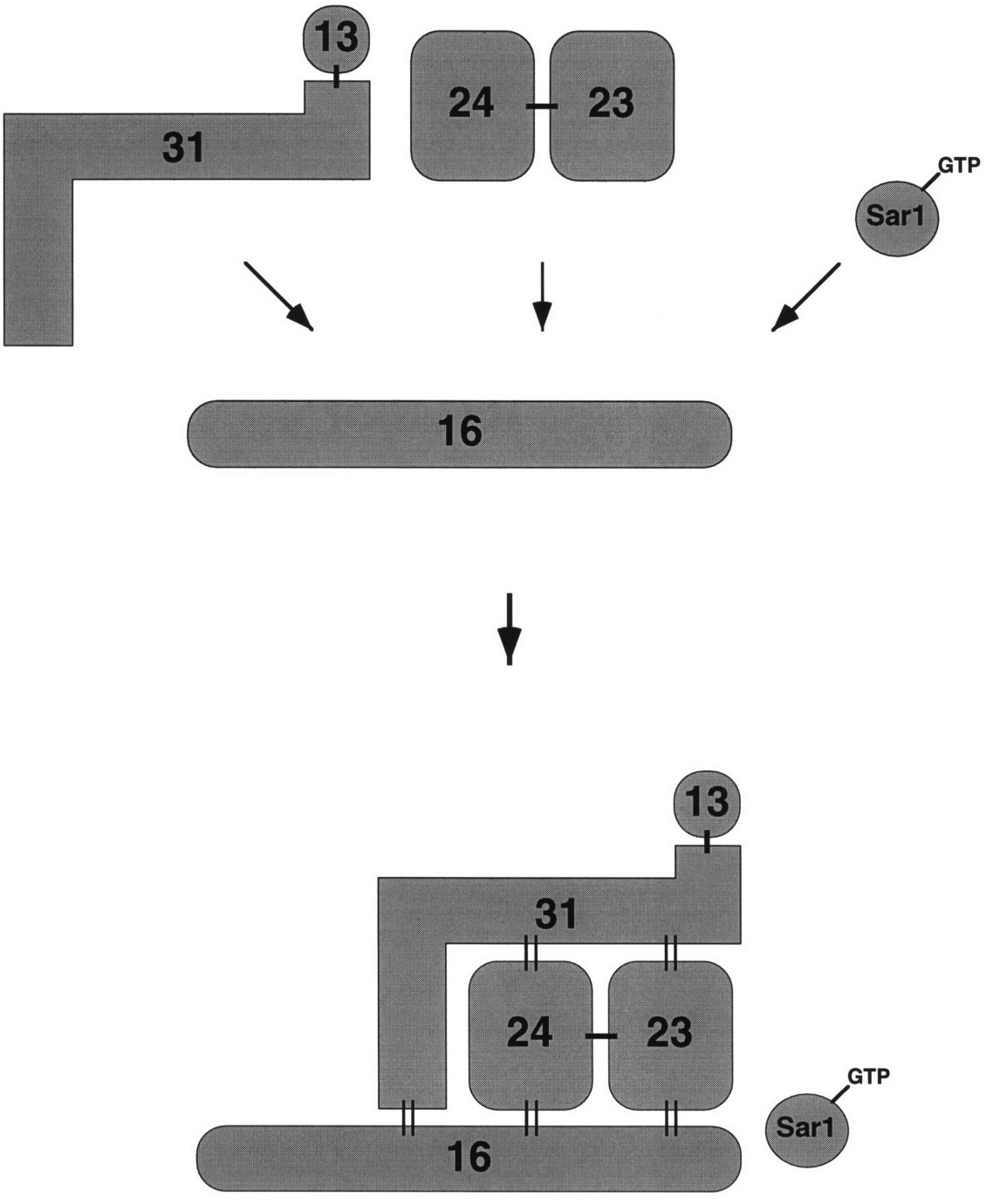

A.
sec31Δ
5-FOA:
–
+
SEC31/CEN
sec31-ΔC/CEN
sec31-ΔC/2μ
Vector
B.
sec31-2
24°C
36°C
C.
SEC31/CEN
sec31-ΔC/CEN
sec31-ΔC/2μ
Vector
0
30'
p2
p1
m
D.
1
2
Sec31p
Sec31ΔCp

Figure 4.

Proposed role of Sec16p in the organization of the COPII coat. Formation of COPII vesicles in vitro requires the presence of three cytosolic components: the Sec31p/Sec13p complex, the Sec23p/Sec24p complex, and the GTP-binding protein Sar1p. Sec16p, which is tightly associated with the cytosolic face of the ER membrane, is proposed to organize the assembly of the COPII coat, binding directly to Sec31p, Sec23p (6), and Sec24p (11). The interaction of Sec31p with Sec23p and Sec24p is also expected to contribute to the assembly and stability of the COPII coat. No attempt at correct stoichiometry has been made. Direct interaction of Sar1p with Sec16p has not been demonstrated.

Figure 4



Chapter V

Prospectus

The work presented in this dissertation represents an attempt to further our understanding of the proteins responsible for the formation of COPII coated vesicles. In yeast, each of the COPII proteins has been shown to be required for ER to Golgi transport. Homologs of three COPII proteins -- Sec13p, Sec23p, and Sar1p -- have been discovered in mammalian cells; each of these homologs has been localized to the cisternae and vesicles in the region of the transitional ER. Mammalian Sec13 and Sar1 have been shown to be required for ER to Golgi transport (mammalian Sec23 was not examined). Collectively, these data emphasize the extent to which the process of COPII-mediated protein transport has been evolutionarily conserved.

Although the individual steps leading to the formation of COPII vesicles remain incompletely understood, we are beginning to define many of the interactions involved in this process. One possible mechanism leading to the formation of the COPII coat is described below.

In this model, the first step in vesicle formation is the recruitment of the small molecular weight GTP-binding protein, Sar1p. This is presumably mediated by Sec12p, an ER membrane protein known to function as a nucleotide exchange factor for Sar1p. Although Sec12p itself is not found in transport vesicles, it has recently been found to associate with Sec16p (P. Espenshade, unpublished results). Given the relatively weak nucleotide exchange activity which has been demonstrated for Sec12p, the existence of a co-factor which would enhance this activity seems a reasonable possiblility, and Sec16p would be well-situated to perform this function.

Although the Sec13p/Sec31p and Sec23p/Sec24p complexes are usually considered to be cytosolic factors, their intracellular location has not been definitively established. For example, Sec23p was initially thought to be a membrane-associated protein, and its release into the cytosolic fraction was

only accomplished by both vigorous lysis and specific pH conditions (Hicke et al., 1989). In our hands, only a very small amount of either of these complexes is present in the cytosolic fraction of cells lysed vigorously in the presence of 50 mM NaCl; increasing the salt concentration to 150 mM increases the recovery of these proteins. Thus, while it is common to speak of the recruitment of the Sec13p/Sec31p and Sec23p/Sec24p proteins from the cytosol (e.g. Schekman and Orci, 1996), these factors may exist as loosely-associated peripheral ER membrane proteins, possibly bound to either transmembrane cargo proteins or cargo adaptors (*vide infra*). Thus, the role of Sar1p may be to initiate the lateral recruitment of the COPII sub-complexes (along with their bound cargo) to Sec16p. Alternatively, the COPII molecules may exist loosely associated with Sec16p itself; perhaps Sar1p alters the conformation of pre-bound complex molecules to a more stable configuration.

A central feature of our model of COPII vesicle formation is the proposed function of Sec16p as a foundation for COPII coat assembly. Evidence in support of this model include:

- *SEC16* interacts genetically with *SEC12*, *SEC13*, *SEC31*, *SEC23*, *SEC24*, and *SAR1*;
- *SEC16* is essential, and is required for ER to Golgi transport;
- Sec16p is tightly associated with the ER membrane, and is found on ER-derived transport vesicles;
- Sec16p binds Sec31p, Sec23p, Sec24p, and Sec13p (see Appendix II);
- *sec16* mutant membranes are impaired for COPII vesicle formation in vitro;
- antibodies to Sec16p inhibit vesicle formation in vitro (P. Espenshade, unpublsihed results).

Assembly of the COPII coat might be facilitated by homotypic interactions between Sec16p molecules (P. Espenshade, unpublished results); the molecules of the COPII sub-complexes might also serve a cross-linking function: for example, Sec23p bound to one Sec16p molecule might be associated with Sec24p bound to a different Sec16p molecule. Similarly, Sec31p bound to one Sec16p molecule could interact with either Sec23p or Sec24p associated with a different Sec16p molecule.

The role of the COPII coat proteins in cargo selection remains an important question. It seems reasonable to envision that the COPII coat might participate in the selection of vesicle cargo, and function as a large "sorting receptor." In the case of transmembrane cargo, this might be accomplished by direct interactions with the COPII coat; the two-hybrid interaction between Sec16p and Chs1p (Appendix I) might represent one example of this type of association. Soluble cargo would require association with an adapter molecule, a transmembrane protein that might interact both with the COPII coat on the cytosolic face of the ER as well as with specific cargo molecules within the ER lumen. Emp24p/Bst2p might function in this capacity, although the direct binding of cargo to this protein has yet to be demonstrated. The possibility that a transmembrane cargo protein might function as an adaptor for soluble cargo should also be considered.

The evolutionary conservation of COPII function affords the opportunity to study a fundamental biological process in several different systems. For example, different, highly-specialized cell-types might employ slightly different COPII proteins; the COPII coat used by a cell devoted to the secretion of insulin might well differ slightly from the coat used by a cell devoted to the secretion of vasopressin. Alternatively, the coats might be identical, but the (proposed) adaptor molecules different. The determination

of the core components involved in this process allows us to examine how this basic theme has been developed and modified in different biological systems; in turn, this knowledge provides a us with a more nuanced understanding of the essential process under investigation.

Our knowledge of ER to Golgi transport has advanced considerably in the seventeen years since the initial *sec* screen was performed. The combination of genetic and biochemical approaches have enabled us to define and purify the proteins required for the in vitro reconstitution of vesicle formation at the ER. However, many unresolved questions remain, including:

- Are there additional proteins (either soluble or membrane-associated) reguired for vesicle formation in vivo? Does Iss20p (Appendix I) represent an example of such a protein?
- What is the step-wise sequence of events leading to the formation of the vesicle coat?
- What is the role played by membrane lipids in both vesicle formation and cargo selection?
- What is the structure of membrane-associated Sec16p?
- What is the role (if any) of Sec16p in the selection of the site of vesicle budding? If Sec16p is involved in this process, how is its recruitment regulated?
- What is the role (if any) of the COPII coat in either initiating membrane deformation or in the scission of the completed vesicle?

Having defined the critical components of COPII vesicle formation machinery, we are now challenged to understand how these components collaborate, and how their function is regulated physiologically.

References

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Appendices

PREFACE

Appendix I and Appendix II extend upon the experiments presented in Chapter IV; the reagent descriptions and reference lists appearing in these sections are intended to complement the information included in the chapter.

Appendix I

Preliminary characterization of the Sec16p-interacting protein Iss20p

OVERVIEW

Three two-hybrid screens were performed in effort to identify proteins that would interact with Sec16p. One of these screens, described in Chapter IV, identified Sec31p as a candidate interactor. Three additional genes were identified by these screens. *CHS1* and *OLE1* were identified in screens for proteins that interact with the C-terminal region of Sec16p (amino acids 1645-2194); ORF YPP049c (also yp9499.07c, and here designated *ISS20* [Interacts with Sec Sixteen]) was identified in a screen for proteins that would interact with the central region of Sec16p (amino acids 447-1235). Both Chs1p and Ole1p have been described previously, and both are associated with the organelles of the secretory pathway. Chs1p encodes a chitin synthase, and has three transmembrane domains; the protein has been localized to both the plasma membrane and to intracellular membrane-bound particles known as chitosomes (Leal-Morales et al., 1994). Chs1p thus represents an example of a cargo protein which transits through the secretory pathway, and might interact with Sec16p during packaging into transport vesicles at the ER. Ole1p, a fatty acid desaturase, has seven potential transmembrane domains, and resides in the ER (Stukey et al., 1990). As such, it might contribute to the anchoring of Sec16p to the ER membrane. Although we have not further pursued the study of these proteins, the role of Ole1p and particularly Chs1p in the recruitment of the COPII coat bears further investigation.

ISS20 represents a novel gene, predicted to encode a 1178 amino acid protein that contains a prominent coiled-coil domain (Fig. 1). Regions within this domain of Iss20p exhibit 17-25% identity to regions within the extensive coiled-coil domain of the general fusion factor Uso1p; this homology thus seems to reflect a shared structural motif, and need not imply a shared

function. The balance of this section describes preliminary attempts to determine the whether *ISS20* is involved in ER to Golgi transport.

PRELIMINARY CHARACTERIZATION OF *ISS20*

ISS20 was isolated in the same screen which identified Sec31p as an interactor with the central region of Sec16p (Chapter IV). The region of Sec16p which binds to Iss20p was subsequently mapped to a 165-amino acid region of Sec16p (amino acids 660-824). This region by itself does not exhibit interactions with Sec23, Sec24p, or Sec31p. The *ISS20* clone isolated represents the C-terminal 812 amino acids of the 1178 amino acid full-length protein identified as the open reading frame YPR049c in the database. The partial *ISS20* clone was used as a probe to identify by hybridization and then isolate the complete genomic locus from a library in pCT3 (Thompson, 1993). Two different pCT3 clones containing the entire *ISS20* ORF were identified, pDS158 (containg 1.5 kb upstream sequence and 6.4 kb downstream sequence) and pDS161 (4.3 kb upstream sequence and 1.0 kb downstream) (see Fig. 1). This determination was based on the sequence analysis of the insert ends, and comparison with the yeast genome database.

To determine if *ISS20* is essential, the genomic locus was disrupted by one-step gene replacement (Rothstein, 1991). Two independent transformation constructs were made. In the first approach (outlined in Fig. 2), fragments representing the 5' and 3' ends of the *ISS20* gene were isolated by restriction digest and cloned into the integrating vector pRS306 in the appropriate orientation, to generate pDS178. The wild-type yeast strains CKY8 and CKY10 were mated, and the resulting diploid strain (*MATa/α ura3-52/ura3-52 leu2,3-112/leu2-3,112*) was transformed with pDS178 that had been

linearized by digestion with NotI. Transformants were selected on media lacking uracil; two transformants were then sporulated; 16 of 20 tetrads exhibited a 2:2 (live:dead) segregation pattern, 3 tetrads had one live spore, and 1 had no live spores. The dead spores in tetrads exhibiting a 2:2 pattern seemed to double 3-4 times before arresting, as 7-15 microcolonies were observed under the microscope. These data were most consistent with the disruption of an essential gene.

To investigate whether *ISS20* was required for ER to Golgi transport, we wanted to generate a strain carrying a chromosomal deletion of *ISS20* covered by a plasmid expressing Iss20p from a galactose-regulated promoter. By examing the transport of the marker cargo protein CPY following the transfer of cells from medium containing galactose to medium containing glucose, the involvement of Iss20p in ER to Golgi transport might be determined. To conduct this experiment, we first needed to construct a *ISS20*-deletion heterozygote that was both *Gal+* and which retained the uracil auxotrophy. To accomplish this, we mated strains CKY263 and CKY264 to generate the diploid strain DSY388 (*MAT* ***a***/α *ura3-52/ura3-52 leu2,3-112/leu2,3-112 Gal+/Gal+*). Next, we designed an integration fragment that utilized the 5' and 3' regions of *ISS20* (generated by PCR) and a *hisG-URA3-hisG* fragment as the selectable marker (see Fig. 3). Growth of strains containing this fragment (obtained by digesting pDS285 with KpnI and NotI) on 5-FOA results in recombination between the *hisG* regions, and loss of the *URA3* gene, thus regenerating the original uracil auxotrophy. Transformants of DSY388 were initially plated on media lacking uracil. Sporulation of two randomly-chosen transformants resulted in a 2:2 (live:dead) segregation pattern. One of these transformants (DSY388) was then grown in the presence of 5-FOA. Segregants were then selected, and found to be Ura-, as expected.

One of these segregants (DSY389) was then spoulated, and again, a 2:2 segregation pattern was observed.

To verify that disruption of the *ISS20* gene was responsible for the observed lethality, pDS161 was transformed into DSY389. Surprisingly, sporulation of the segregants revealed a 2:2 pattern, in 10/10 tetrads examined. These results are discussed below.

We also attempted to demonstrate a role for Iss20p in secretion using an approach recently developed by the laboratory of K. Struhl (Moqtaderi et al., 1996). In this "double-shutoff" system (Fig. 4), the gene to be studied is expressed as a fusion with ubiquitin and the hemagglutin (HA) epitope, and is placed under the control of a Rox1p-repressible promoter (Zitomer and Lowry, 1992). Integration of this fusion constructed is directed to the locus of the gene to be studied, thus disrupting it, and ensuring that the only source of the gene is the integrated fusion construct. A strain is utilized in which both *UBR1* and *ROX1* are placed under the control of a promoter regulated by Ace1p (Furst and Hamer, 1989). Addition of copper to the growth medium results in the rapid expression of both *UBR1* and *ROX1*, and the subsquent repression of the target gene as well the ubiquitin-mediated degradation of its protein product (Johnson et al., 1992). As a result of the addition of copper, the gene under examination is transcriptionally repressed and the protein product is degraded. In tests of this system transcription factors have been shown to be reduced to only 1% of their steady-state level in two hours. After Iss20p depletion, pulse-chase analysis of CPY transport could then be used to evaluate the effect on secretion.

Generation of the integration plasmid required a 5' region of *ISS20* that containing a unique site which could be used to linearize the integration plasmid. A 120 bp 5' region of *ISS20* was amplifed by PCR; this fragment,

which contained a centrally-located PstI, was inserted into pZM168, to generate pDS240. This plasmid was linearized with PstI, and transformed into ZMY60 (equivalent to DSY373). The transformation efficiency appeared to be quite low, possibly reflecting the small size of the regions (approximately 60 bp each) required for recombination. However, several transformants were obtained. All the transformants were able to grow on selective media containing copper, suggesting that if the fusion construct was functioning as designed, then the fusion protein was not essential for cell viability.

In an attempt to increase the transformation efficiency, another fusion plasmid (pDS264) was constructed that utilized a longer fragment of *ISS20*, also generated by PCR. Again, the transformation efficiency of ZMY60 appeared quite low, but several transformants were obtained. All the transformants tested were again able to grow on selective media containing copper. To test for the expression of the fusion protein, extracts were prepared from either ZMY60 transformants or from the orginal ZMY60 strain. Proteins were separated by SDS-PAGE, and analyzed by immunoblotting using anti-HA antibodies. A protein of the predicted size was present in the transformant extracts, but not the control ZMY60 extracts, suggesting that the construct had integrated in the expected location. The effect of copper induction on the expression of the fusion protein has not yet been investigated.

The simplest explanation for these results is that *ISS20* is not an essential gene, and that the disruption of *ISS20* is lethal because it disrupts an important regulatory region of a neighboring gene. Presumably, this neighboring gene is not entirely represented on the pCT3 clone used to assess complementation. The best candidate gene is ORF YPR048W (also yp9499.06w). The 3' end of this ORF (predicted to encode a NADPH-

cytochrome p450 reductase) is less than 100 bp away from the 3' end of the Iss20p coding sequence, and thus, it is possible that disruption of the *ISS20* locus might also corrupt regulatory elements required for proper expression of the YPR048W gene. Since this gene is approximately 2 kb in length, it would not be encompassed by the pDS161 clone, explaining why this clone did not complement the *ISS20* disruption. If this explanation is correct, then transformation with pDS158, which contains 6.4 kb of sequence 3' of the *ISS20* gene, should rescue the lethality. Southern blot analysis could also be performed to confirm disruption of the *ISS20* gene.

In summary, while the interaction of Iss20p with Sec16p raises the possibility that Iss20p is involved in ER to Golgi transport, as yet we have not been able to obtain confirmatory evidence to support this hypothesis, and further studies will be required to clarify the role of this novel protein.

MATERIALS AND METHODS: construct generation

pDS178. pDS161 was digested with KpnI and Bam to liberate a 5.6 kb fragment spanning the *ISS20* locus. This fragment was inserted into pKS(+) to generate pDS167. pDS167 was then digested with PstI, followed by reaction with klenow fragment to generate a blunt end. The DNA was then digested with SpeI to generate a 640 bp fragment representing the 121 bp of the *ISS20* coding sequence. This fragment was cloned into pKS(+) that had been digested with ClaI, reacted with klenow to blunt the end, then digested with SpeI; the resulting plasmid, designated pDS177, was then digested with NotI and XhoI, and the insert (representing the extreme 5' region of *ISS20*) recovered. pDS167 was digested with SacI and NotI to obtain a fragment representing the extreme 3' region of *ISS20*. A three-part ligation was then performed using

these two inserts and a fragment obtained by digesting pRS306 with SacI and XhoI. The resulting plasmid, designated pDS178, was linearized with NotI prior to transformation.

pDS285. pDS226 represents the 5.6 kb BamHI/KpnI *ISS20* fragment cloned into the pRS316 vector. To generate fragments reprsenting the 5' and 3' regions of *ISS20*, PCR reactions were performed using pDS226 as the template, and the following primers: Reaction I: sense primer: Stratagene T3 sequence; anti-sense primer: 5'-AAAGATCTTTAATCAGCGTCTGCCATGATG-3'. Reaction II: sense primer: 5'-AAAGATCTGGAGTTTGAACAGTGCTAAAC-3'; antisense primer: Stratagene T7 sequence. Reaction I fragment was digested with KpnI/BglII, reaction II fragment was digested with BglII/NotI, and pKS(+) was digested with KpnI/NotI; a three-part ligation was then performed to generate pDS284. A 3.8 kb BamHI/BglII fragment containing the *hisG-URA3-hisG* sequence was recovered by digestion of pNKY51 (Alani et al., 1987), generously provided by N. Kleckner. This fragment was inserted into pDS284 that had been digested with BglII, to generate pDS285. The 6 kb KpnI/NotI insert was used for transformation.

pDS240. pDS161 was used as a template for a PCR reaction using the following primers: sense: 5'-AAGAATTCCATGGCAGACGCTGATG-3'; antisense: 5'-AAGCGGCCGCAAATCAACATTCGTAG-3'. This PCR fragment was digested with R1/NotI, and inserted into pZM168 (digested with the same enzymes). The resulting plasmid was then digested with KpnI/NotI, and the recovered 1kb fragment was inserted into pRS404, to generate pDS240. This plasmid was linearized with PstI prior to transformation.

pDS264. pDS167 was used as a template for a two PCR reactions using the following primers: reaction I, sense: 5'-AAGAATTCCATGGCAGACGCTGATG-3'; antisense: 5'-GGTCTAGAGTAAAAGACCTATTA-3'; reaction II, sense: 5'-GGTCTAGATGATTTTTACGTTTAC-3'; antisense: 5'-GGCGGCCGGATATATGACTATTC-3'. These PCR fragments were digested, respectively, with EcoRI/XbaI and XbaI/EagI, and inserted into pZM168 (digested with EcoR1 and EagI), to generate pDS263. This plasmid was then digested with KpnI/SacI, and the 1.3 kb recovered fragment was inserted into pRS306. This plasmid was linearized with XbaI prior to transformation.

ACKNOWLEDGEMENTS

The double-shutoff system was developed by Zarmik Moqtaderi; her generosity with both reagents and suggestions has been appreciated.

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FIGURES

Figure 1

(**A**) Map of *ISS20* (YPR049C) genomic locus. Arrows indicate orientation of ORFs. YPR048W represents a neighboring ORF encoding a protein with homology to the NADPH-cytochrome p450 reductase. *MAK3* encodes an N-acetyltransferase required for the propogation of the L-A doubled-stranded RNA virus (Tercero and Wickner, 1992). pDS158 and pDS161 represent pCT3 clones (Thompson et al., 1993) containing the indicated genomic regions.

(**B**) Diagram of Iss20p, indicating the location of the coiled-coil domain (Paircoil sequence analysis program, Berger et al., 1995) . The region of Iss20p encoded by the library clone isolated in the two-hybrid screen for Sec16p (amino acids 447-1235)-interactors is also shown.

Figure 1A

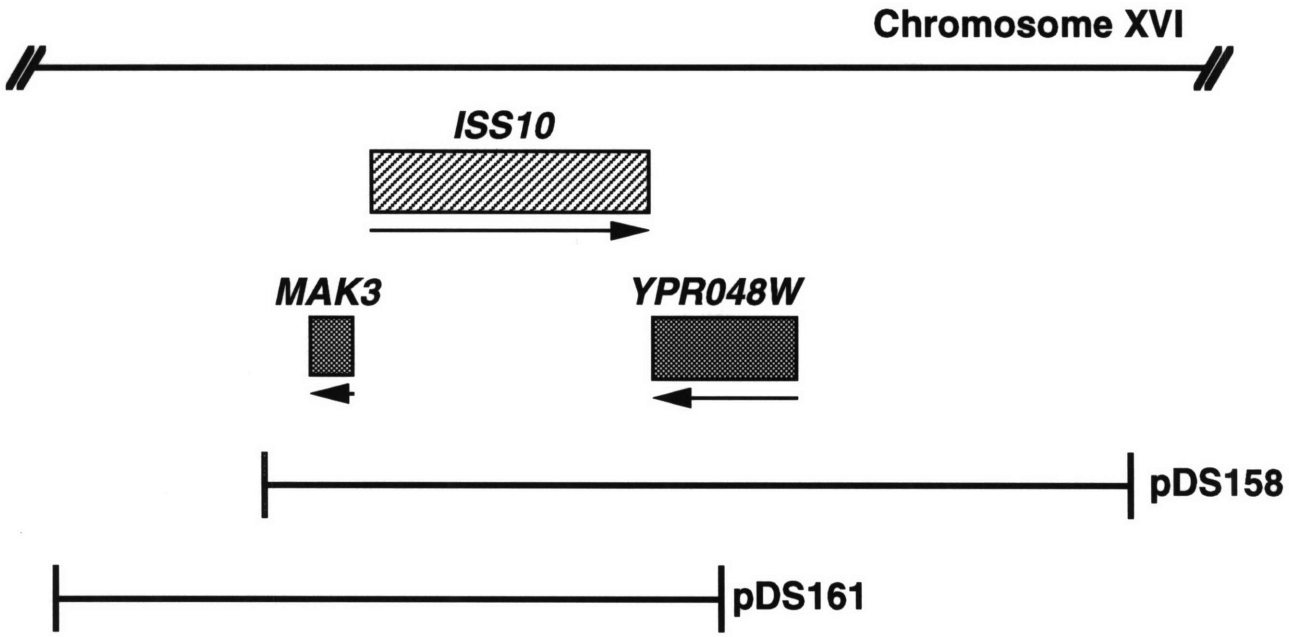


Figure 1B

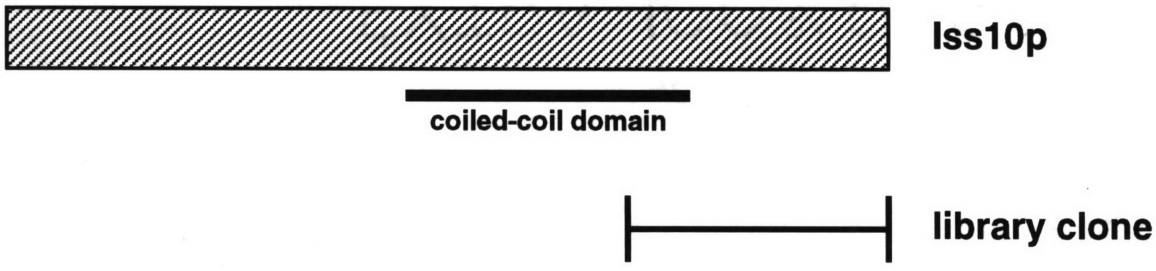


Figure 2

Gene disruption of *ISS20* using pDS178. See text for details.

Figure 2

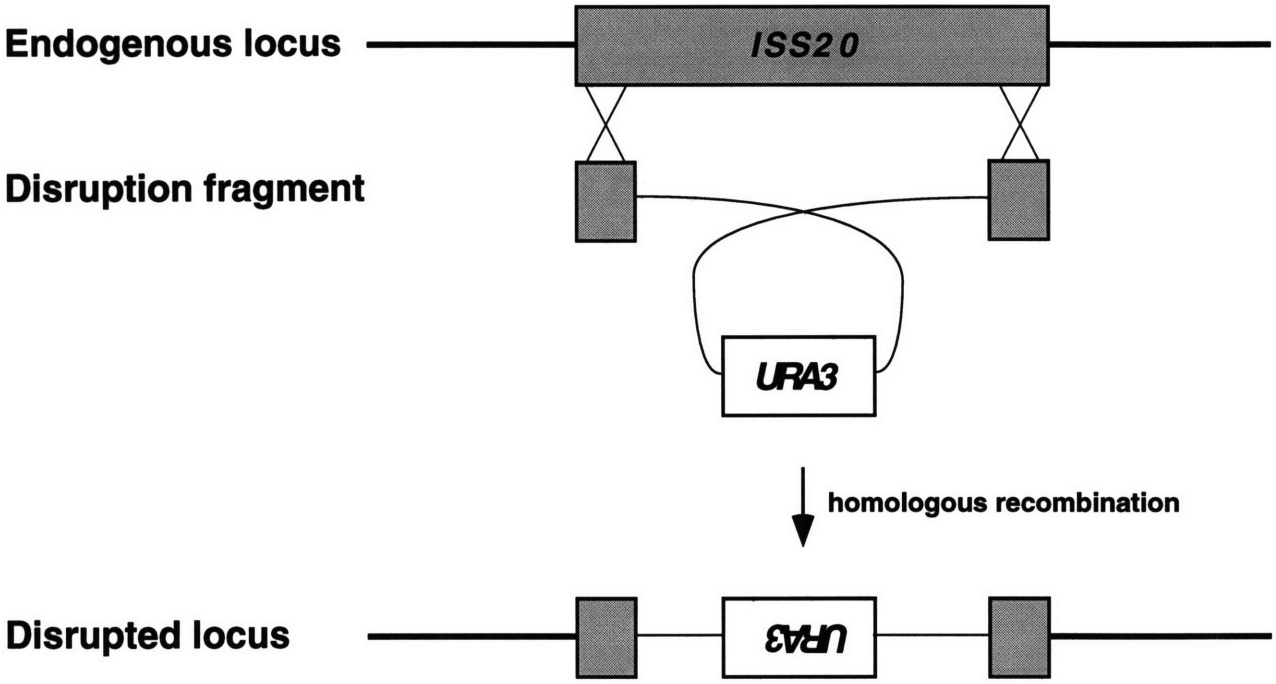

Endogenous locus
ISS20
Disruption fragment
URA3
homologous recombination
Disrupted locus
URA3

Figure 3

Gene disruption of *ISS20* using fragment excised from pDS285; see text for details.

Figure 3

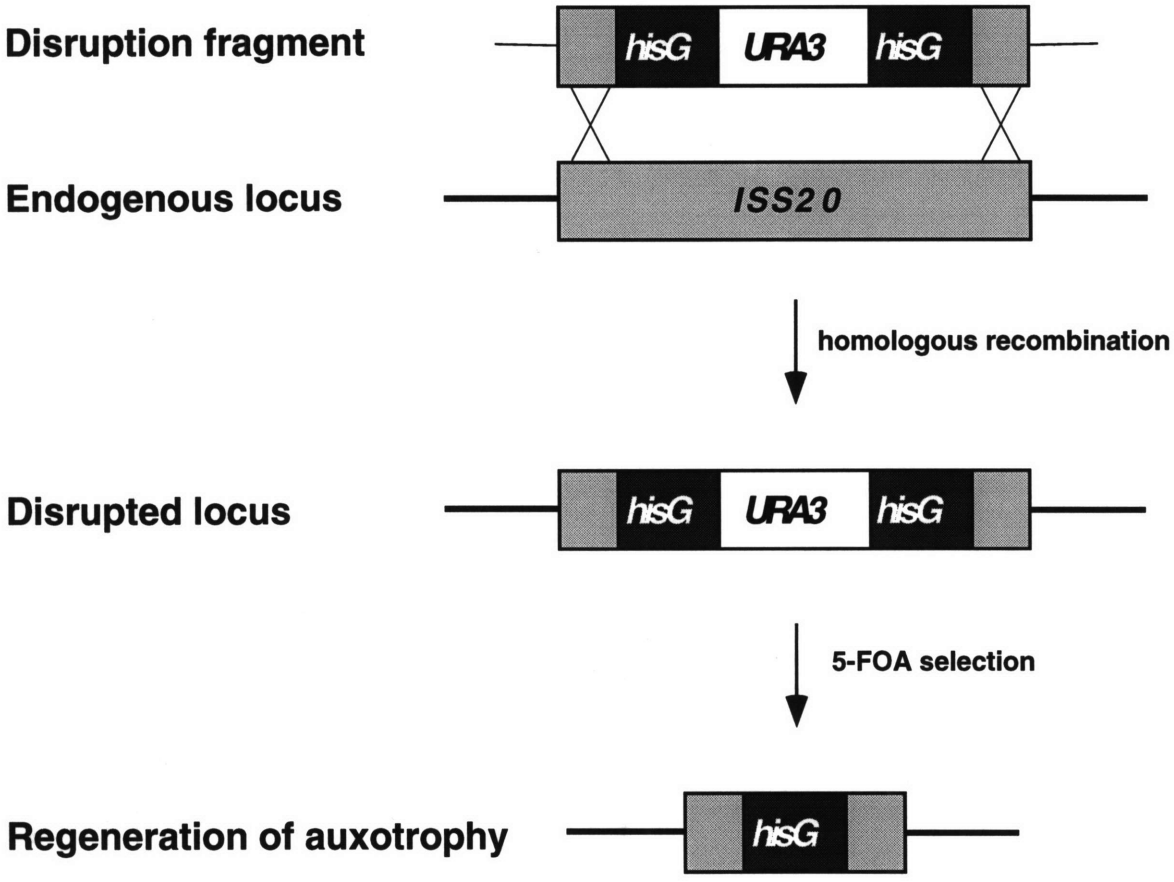
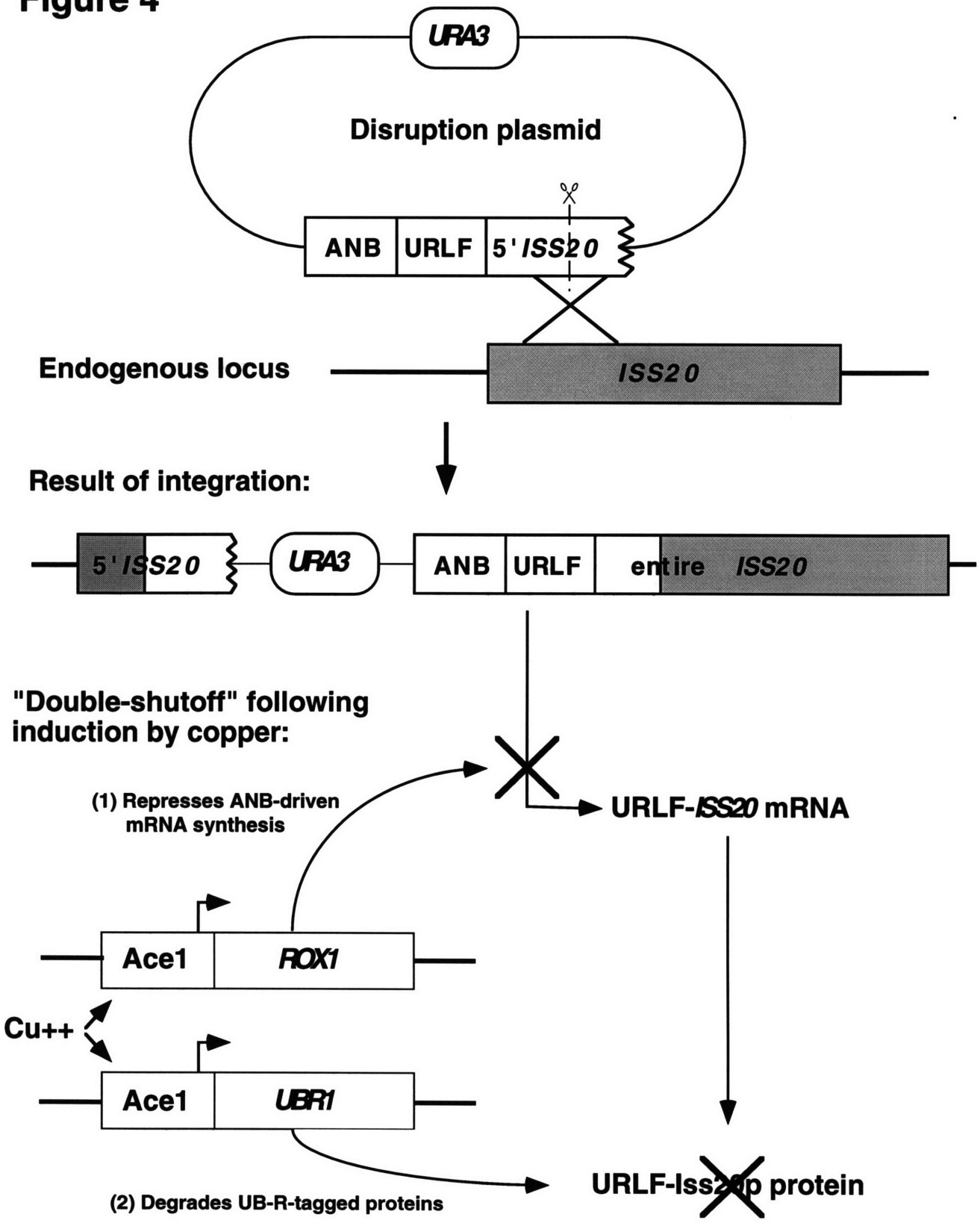

Disruption fragment
hisG
URA3
hisG
Endogenous locus
ISS2 0
homologous recombination
Disrupted locus
hisG
URA3
hisG
5-FOA selection
Regeneration of auxotrophy
hisG

Figure 4

Outline of double-shutoff system. The system consists of two parts: (1) a parent strain (ZMY60) containing copper-inducible alleles of *ROX1* and *UBR1*; (2) a short 5' region of *ISS20* fused in frame with a ubiquitin-arginine-lacI-HA ("URLF") cassette, and regulated by the *ANB1* promoter; this fusion construct is inserted into an integrating vector. The resulting "disruption plasmid" -- in this case, either pDS240 or pDS264 -- is linearized at a site within the *ISS20* fragment, and integrated by homolgous recombination as shown. (Note that full-length HA-tagged Iss20p will only be produced if the disruption plasmid integrates in the proper location.) When the strain containing the correctly-integrated plasmid is induced with copper, transcription of the fusion product is repressed by Rox1p, while Ubr1p mediates the degradation of the residual URLF-Iss20p protein. See text for details.

Figure 4


URA3
Disruption plasmid
ANB
URLF
5' ISS20
Endogenous locus
ISS20
Result of integration:
5' ISS20
URA3
ANB
URLF
entire ISS20
"Double-shutoff" following induction by copper:
(1) Represses ANB-driven mRNA synthesis
URLF-ISS20 mRNA
Ace1
ROX1
Cu++
Ace1
UBR1
URLF-Iss20p protein
(2) Degrades UB-R-tagged proteins

Appendix II

Two-hybrid interactions of Sec16p with Sec31p, Sec24p, and Sec13p

SUMMARY

Sec31p and Sec24p have both been shown to bind to the central region of Sec16p (amino acids 447-1293). In an attempt to map the binding sites more precisely, the interaction of Sec31p, Sec24p, and Sec13p with different regions of Sec16p was evaluated by two-hybrid analysis. We report the identification of regions of Sec16p that interact specifically with each of the three proteins investigated.

RESULTS AND DISCUSSION

Sec24p and Sec31p were both previously shown to bind the central region of Sec16p (amino acids 447-1235). In an effort to determine more precisely where Sec24p and Sec31p bound Sec16p, protein fragments representing different regions within the central domain of Sec16p were expressed as LexA-fusions. These constructs were transformed into CKY556 and evaluated by two-hybrid analysis against the following proteins, represented as activation domain-fusions: Sec24p (C-terminal region, amino acids 666-926); Sec31p (C-terminal region, amino acids 1147-1273); and Sec13p.

The results of this analysis (Fig. 1) suggest that specific regions within the central domain of Sec16p interact with Sec31p, Sec24p, and Sec13p. Interpretation of these data is complicated both by the high background associated with the pDS293-transformants, and by the weak association observed between pDS143 and the Sec24p constructs (though this association was consistently found to be above background).

These data represent the first evidence that Sec13p directly associates with Sec16p, and lend additional support to our hypothesis that Sec16p serves as a foundation for the assembly of the COPII coat (Fig. 2).

MATERIALS AND METHODS: plasmids used in two-hybrid analysis

LexA-fusion plasmids (vector: pBTM116):

pDS293 (Sec16p, amino acids 447-700).

pDS143 (Sec16p, amino acids 661-824).

pDS144 (Sec16p, amino acids 877-1235).

Activation domain-fusion plasmids (vector: pGADGH):

2a8 (Sec31p, amino acids 1147-1273).

pDS271 (Sec24p, amino acids 666-926).

pDS141 (Sec13p).

pDS102 (vector only).

Figures

Figure 1

Dissection of Sec16p binding domains through the use of two-hybrid analysis. To define more precisely the binding domains within the central region of Sec16p (amino acids 447-1235), a series of Sec16p truncations were constructed in the LexA-fusion vector pBTM116: pDS293 (Sec16p amino acids 447-700); pDS143 (amino acids 661-824); pDS144 (amino acids 877-1235). These plasmids were evaluated against a series of activation domain-fusions to *SEC31* (coding sequence for amino acids 1147-1273), *SEC24* (coding sequence for amino acids 666-926), and *SEC13* for expression of a *lacZ* reporter. Regions of the central domain of Sec16p that contain charge-rich and proline-rich sequences are indicated in the diagram, as is the region containing the known $sec16^{ts}$ mutations.

Figure 1

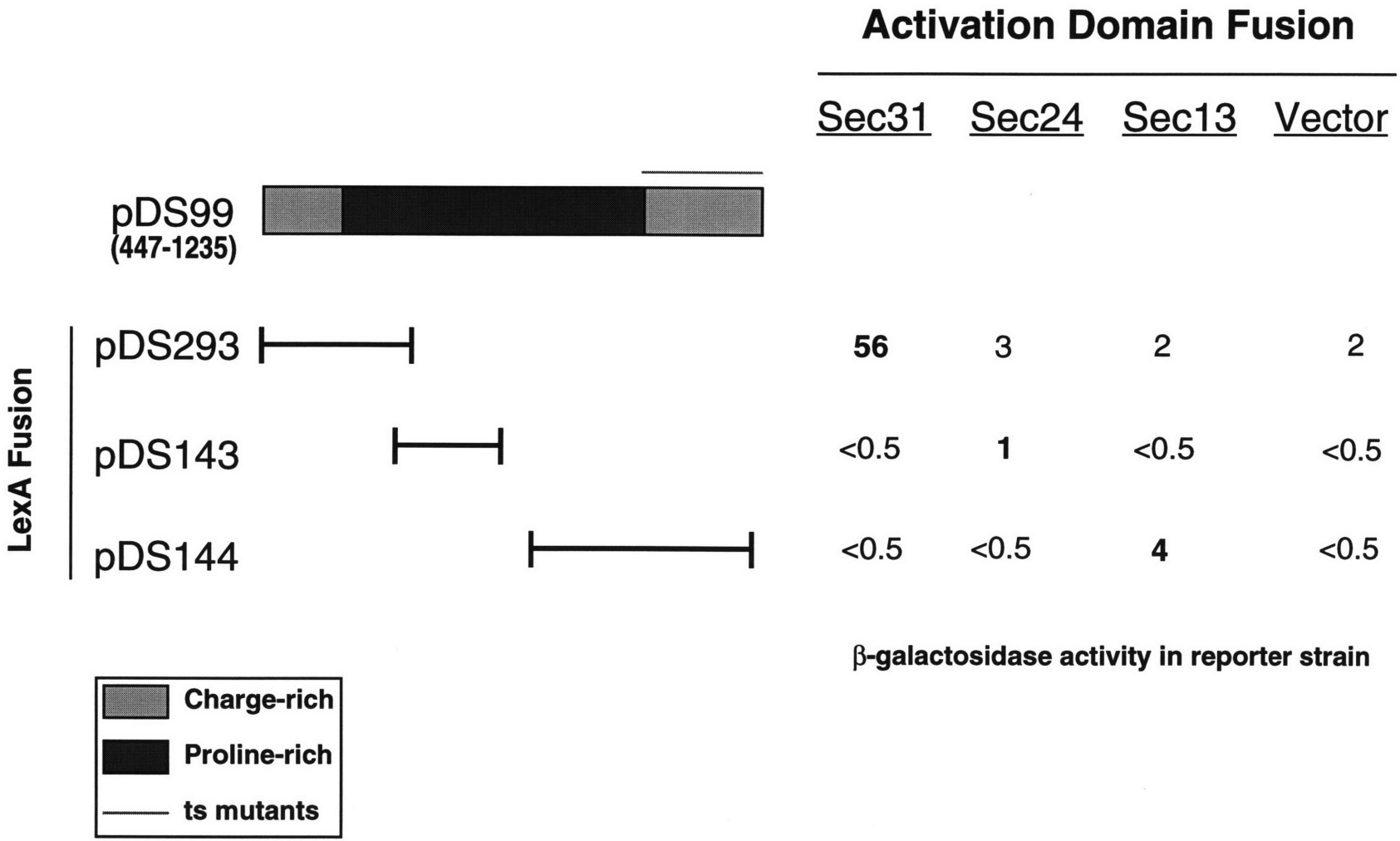
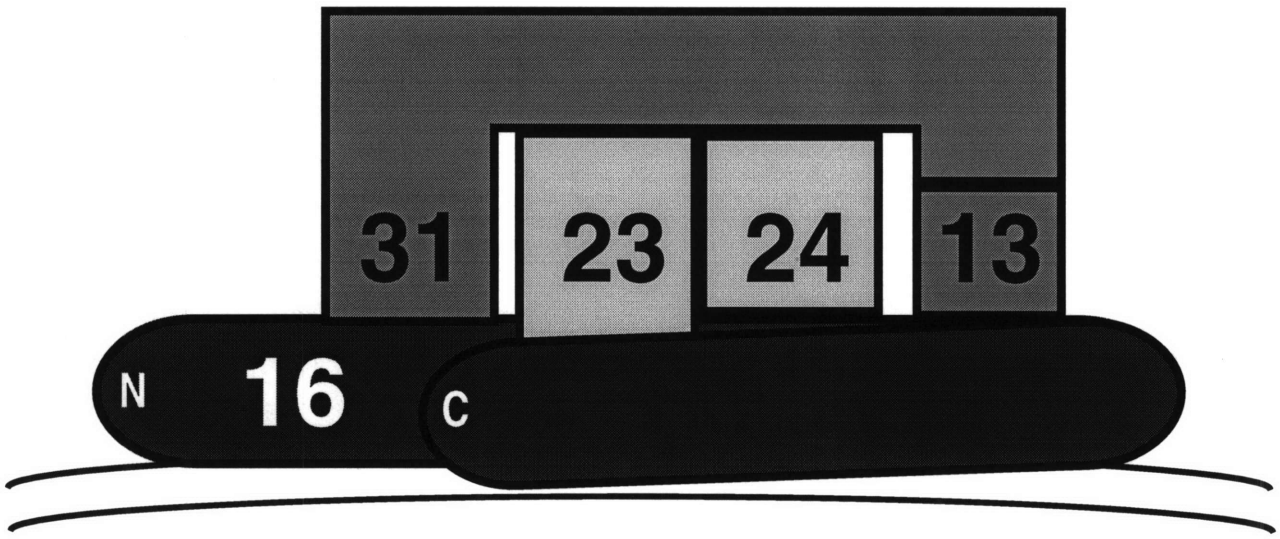

Activation Domain Fusion
Sec31
Sec24
Sec13
Vector
pDS99
(447-1235)
LexA Fusion
pDS293
56
3
2
2
pDS143
<0.5
1
<0.5
<0.5
pDS144
<0.5
<0.5
4
<0.5
β-galactosidase activity in reporter strain
Charge-rich
Proline-rich
ts mutants

Figure 2

Two models of COPII assembly upon Sec16p, reflecting all interactions detected by two-hybrid analysis. See text for details.

Figure 2a



ER Lumen

Figure 2b