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

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

INTRODUCTION

Transport of proteins between compartments of the secretory pathway is mediated by cytoplasmic vesicles that form by budding from the membrane of the donor compartment. In the best-studied cases, vesicle formation is driven by the assembly of vesicle coats that are composed of particular sets of peripheral membrane proteins (reviewed, Schekman and Orci, 1996). COPII-coated vesicles mediate transport from the endoplasmic reticulum (ER) to the Golgi apparatus in both yeast and mammalian cells (Barlowe *et al.*, 1994; Schekman and Orci, 1996). Genetic and biochemical studies in yeast have identified six proteins that are components of the COPII coat and that are required for vesicle formation in vivo and in vitro (Hicke *et al.*, 1989, 1992; Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990; Salama *et al.*, 1993; Barlowe *et al.*, 1994). Five of these proteins—two het-

eromeric protein complexes, Sec13p/Sec31p and Sec23p/Sec24p, and a small GTP-binding protein Sar1p—are found in the cytosol and in association with membranes. Vesicle formation can be reconstituted by adding these five proteins in soluble form to ER membranes that have been stripped of peripheral proteins by extraction with urea (Barlowe *et al.*, 1994). The sixth essential COPII protein, Sec16p, is a 240-kDa peripheral membrane protein that is found on membranes, but not in soluble form, and resists extraction by urea. In an in vitro budding reaction, Sec16p is incorporated into the vesicle coat starting as a peripheral ER protein, not as a soluble protein (Espenshade *et al.*, 1995).

Clearly, an important aspect in COPII vesicle formation is the recruitment of cytosolic coat components to the membrane. By analogy to the role of the small GTP-binding protein ARF in recruitment of the coatomer (COPI) coat to Golgi vesicles (Donaldson *et al.*, 1992), the small GTP-binding protein Sar1p has been proposed to regulate recruitment of Sec23p/Sec24p and Sec13p/Sec31p to the ER membrane (Schekman and Orci, 1996). Membrane-associated re-

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

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

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

MATERIALS AND METHODS

General Techniques

Yeast transformations were performed by standard methods (Kaiser *et al.*, 1994). DNA manipulations were performed as described (Sambrook *et al.*, 1989). For PCR, Vent polymerase was used (New England Biolabs, Beverly, MA). Western blotting was performed as described (Gimeno *et al.*, 1995) with the following antibodies: anti-invertase (1/1000), anti-Sec23p (1/500; kindly provided by L. Hicke and R. Schekman, University of California, Berkeley, CA), anti-Sec24p (1/1000; kindly provided by T. Yoshihisa and R. Schekman, University of California, Berkeley, CA), anti-GST (1/1000; Santa Cruz Biotechnology, Santa Cruz, CA), and HRP-coupled sheep anti-rabbit immunoglobulin (Ig; 1/10,000; Amersham, Arlington Heights, IL). Unless otherwise noted, protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA; Boehringer Mannheim, Indianapolis, IN) as a standard.

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

A temperature-sensitive allele of *SEC24* was isolated by screening a collection of 1800 random temperature-sensitive mutants for accumulation of the ER forms of carboxypeptidase Y and invertase by Western blotting (Chitouras, Frand, and Kaiser, unpublished data). One of these mutants, EH382, displayed a strong, temperature-dependent accumulation of the ER forms of CPY and invertase. This secretion defect segregated 2:2 in crosses and cosegregated with the

temperature-sensitive growth defect, indicating that both growth and secretion defects were caused by a single mutation. The gene responsible for the defects was cloned by complementation of the temperature-sensitive growth defect of EH382 with the use of a library of yeast genomic DNA in pCT3 (Thompson *et al.*, 1993). One complementing clone, pAF70, contained a 4.3-kilobase (kb) insert. Comparison of the restriction map and DNA sequence of pAF70 with the sequence of *SEC24* (generously supplied by T. Yoshihisa and R. Schekman) showed that pAF70 contained the entire *SEC24* open reading frame flanked by 0.8 kb at the 5' and 3' end each. To demonstrate that the mutation in EH382 was indeed a mutation in *SEC24*, we marked the chromosomal *SEC24* locus of a wild-type strain with *URA3* by directing integration of the plasmid pRH285, containing a 3-kb *Bam*HI fragment with the C-terminal 896 amino acids of *SEC24*, to the homologous chromosomal locus by cleaving pRH285 in the insert sequences with *Bgl*II before transformation. The marked strain was then crossed to EH382. Tetrad analysis showed complete linkage of the plasmid sequences to the Ts growth phenotype. The *SEC24* allele in EH382 was designated *sec24-1*.

Two-Hybrid Protein–Protein Interaction Assay

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

Plasmids (Table 1) were constructed as follows. A *SEC24* fusion to *lexA* lacking the N-terminal 33 amino acids (pRH286) was constructed by inserting the 3-kb *Bam*HI fragment of pAF70 into pEG202. To create a full-length *SEC24* fusion to *lexA* (pRH287), the N terminus of *SEC24* was amplified by polymerase chain reaction (PCR) and ligated into pEG202, creating pRH284. The C terminus of *SEC24* was then inserted by ligating the 3-kb *Bam*HI fragment of pAF70 into pRH284. The N-terminal *SEC24* *lexA* fusion (pRH321) contains the 2-kb *Eco*RI fragment of pRH287 ligated into pEG202. The fusion of the C-terminal region of *SEC24* to *lexA* (pRH347) was made by first inserting the 0.6-kb *Eco*RI fragment of pRH287 into pEG202-AAT, a derivative of pEG202, and then inserting the 1.1-kb *Apa*I–*Xho*I fragment of pRH286 into the resulting construct. To make a *GAL1*-promoted *SEC23* fusion to *lexA* (pDS79), we inserted the *Eco*RI–*Xho*I fragment of pPE82 (Espenshade *et al.*, 1995) into pGILDA, a *GAL1*-promoted derivative of pEG202 (kindly provided by D. Shaywitz, Massachusetts Institutes of Technology, Cambridge, MA). The central domain of Sec16p (aa 565–1235) was fused to the *GAL4* acidic activation domain by inserting the *Bam*HI fragment of pPE53 (Espenshade *et al.*, 1995) into pGAD GH (Hannon *et al.*, 1993) to make pPE167. A *lexA* fusion containing aa 447–1235 of Sec16p (pPE62) was made by inserting the *Nco*I–*Pvu*II fragment of pPE4 (Espenshade *et al.*, 1995) into pEG202.

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

Coprecipitation of GST-Fusion Proteins from Yeast Extracts

Glutathione-S-transferase (GST) fusions for coprecipitation experiments were constructed as follows. *SEC24* (pRH305; aa 34–926) was

Table 1. Plasmids

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

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

fused to the *GST* gene expressed from the *GAL1* promoter by inserting a 3-kb *Bam*HI fragment of pAF70 into pPE127, a derivative of pRD56 (Espenshade *et al.*, 1995). To construct a *GST* fusion to the N terminus of *SEC24* (pRH348; aa 34-666), we inserted a 2-kb *Bam*HI-*Eco*RI fragment of pRH321 into pPE127. A *GST* fusion to the C terminus of *SEC24* (pRH360; aa 666-926) was created by first inserting the 0.6-kb *Eco*RI fragment of pRH286 into pPE127, cutting the resulting plasmid with *Bgl*II and *Xho*I, and ligating it with the 1-kb *Bgl*II-*Xho*I fragment of pRH305. To produce a soluble and detectable central domain of Sec16p (pRH317), we fused invertase to amino acids 565-1235 of Sec16p, and the fusion protein was expressed from the *GAL1* promoter. pRH317 is identical to pPE53 (Espenshade *et al.*, 1995), with the exception that the vector backbone is marked with *LEU2*. To facilitate detection of Sec23p, we expressed *SEC23* from the *GAL1* promoter (pPE123).

Coprecipitation experiments were performed in CKY473 (*Mata*, *ura3-52*, *leu2-3*, 112, *Gal*⁺). Cells were grown to exponential phase in selective medium containing 2% raffinose; galactose was added to 2%, and extracts were prepared 3-4 h later. In all, 10-16 $\times 10^7$ cells (5-8 OD₆₀₀ units) were suspended in 30 μ l of coimmunoprecipitation (CoIP) buffer (20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid [HEPES], pH 6.8, 80 mM potassium acetate, 0.2 M NaCl, 5 mM magnesium acetate, 0.02% Triton X-100, and 5 mM dithiothreitol [DTT]) containing a protease inhibitor cocktail (2.5 $\times 10^{-4}$ U/ml α_2 -macroglobulin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 μ g/ml leupeptin, 0.7 μ g/ml pepstatin, and 2 μ g/ml

aprotinin; all Boehringer Mannheim). Cells were lysed by vigorous agitation with glass beads 4 $\times$ 20 sec with 1 min intervals on ice. Extracts were diluted with 200 μ l of CoIP buffer as above, and the lysate was cleared by centrifugation at 13,000 $\times g$ for 10 min, followed by centrifugation at 100,000 $\times g$ for 30 min. The supernatant was diluted to 700 μ l with CoIP buffer (supplemented as above), glutathione-Sepharose 4B beads (Pharmacia, Piscataway, NJ) were added, and samples were incubated for 1 h at 25°C. The beads were washed four times with CoIP buffer, and bound proteins were released by boiling in 30 μ l of extract sample buffer (ESB) (60 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, and 0.001% bromophenol blue).

Preparation of Recombinant Sec23p and Sec24p

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

Fusion proteins were induced and purified as described (Smith, 1993) with the following modifications. Cultures were grown to exponential phase at 30°C for GST-Sec23p and at 25°C for GST-Sec24p; fusion proteins were induced by addition of 0.5 mM isopropyl- β -D-thiogalactoside (IPTG), and growth was continued for 2–3 h. Cells were lysed by sonication in phosphate-buffered saline (PBS) containing 0.5 mM EDTA and the same protease inhibitor cocktail used for the coprecipitation experiments. After addition of 1% Triton X-100, cell debris was removed by centrifugation at $16,000 \times g$ for 10 min at 4°C. Fusion proteins were isolated by batch purification with glutathione-Sepharose 4B beads (Pharmacia). Sec23p and Sec24p were liberated from the GST moieties by cleavage of the bound fusion proteins with thrombin (Sigma, St. Louis, MO) in cleavage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2.5 mM CaCl₂). Cleavage was stopped by addition of 1 mM PMSF. Recombinant proteins were stored in 10% glycerol at –80°C. Protein concentration was determined by comparison of purified protein to dilutions of BSA (Boehringer Mannheim) after resolution by SDS-PAGE and staining with Coomassie brilliant blue (BRL Life Technologies, Gaithersburg, MD).

Binding of recombinant Sec24p to recombinant Sec23p was assayed as follows: 250 μ l of bacterial lysate containing GST-Sec23p (in PBS and 0.02% Triton X-100; total protein, 0.5 mg) was incubated with 30 μ l of packed glutathione beads for 1 h at 4°C. Beads were collected by centrifugation and washed two times with 1 ml of PBS and 0.02% Triton X-100 and once with 1 ml of binding buffer (25 mM HEPES-KOH, pH 6.8, 0.1% Triton X-100, 1 mM MgCl₂, 0.5 mM DTT, and 0.25 mg/ml BSA). Washed beads contained ~11 pmol GST-Sec23p, as judged by Western blotting with GST as a standard. Beads were resuspended in 35 μ l of binding buffer; 6 pmol of Sec24p in 15 μ l cleavage buffer was added. As a control, Sec24p was added to 30 μ l of packed glutathione beads not preincubated with GST-Sec23p. Reactions were incubated at 22°C for 1 h, the beads were collected by centrifugation, and the supernatant containing unbound Sec24p was mixed with an equal volume of 2 $\times$ ESB. Beads were washed two times with 1 ml of binding buffer and solubilized by the addition of 100 μ l of 1 $\times$ ESB. Equal volumes of protein samples were separated by SDS-PAGE and visualized by staining with Coomassie brilliant blue (BRL).

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

The central and C-terminal domain of Sec16p (amino acids 565–1235 and 1645–2194, respectively) were expressed in yeast as GST-fusion proteins from the *GAL1* promoter (pRH323 and pPE122, respectively). pRH323 is the 2-kb *Bam*HI fragment of pPE53 (Espenshade *et al.*, 1995) in pPE127. pPE122 has been described (Gimeno *et al.*, 1995). As a control, GST (no fusion) was expressed from the *GAL1* promoter (pRD56; Espenshade *et al.*, 1995).

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

For binding experiments, aliquots of the frozen supernatants were mixed with 30 μ l of packed glutathione-Sepharose beads (Pharmacia) in a total volume of 500 μ l of lysis buffer. Beads were incubated for 1 h at 4°C and then collected by centrifugation. The supernatant was discarded, and the beads were washed two times with 1 ml of lysis buffer, once with lysis buffer lacking NaCl (20 mM HEPES-KOH, pH 6.8, 80 mM KOAc, 5 mM MgOAc, and 0.02% Triton X-100), and once with binding buffer (25 mM HEPES-KOH, pH 6.8,

0.1% Triton X-100, 1 mM MgCl₂, 0.5 mM DTT, and 0.25 mg/ml BSA). Beads were resuspended in 35 μ l of binding buffer to which the indicated amount of recombinant Sec23p or Sec24p in 15 μ l of cleavage buffer was added. Unless otherwise indicated, the final concentration of NaCl in the reaction was 45 mM. Reactions were incubated for 1 h at 22°C; beads were then collected by centrifugation, and unbound recombinant Sec24p or Sec23p was sampled by mixing 20 μ l of supernatant with an equal volume of 2 $\times$ ESB. Beads were washed two times with 1 ml of binding buffer, and bound proteins were solubilized by addition of 100 μ l of 1 $\times$ ESB (GST and GST-Sec16p central domain). Samples that contained GST fused to the C-terminal domain of Sec16p were first treated with 0.1 μ g of thrombin in 50 μ l of cleavage buffer for 1 h and then solubilized by adding an equal volume of 2 $\times$ ESB. Thrombin digestion was necessary to distinguish Sec24p from GST-Sec16p (aa 1638–2194), because these two proteins migrated similarly on gels, and the anti-Sec24p antibody cross-reacted with the GST-fusion protein. Equal volumes of bound and unbound proteins were resolved by SDS-PAGE and analyzed by Western blotting.

RESULTS

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

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

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

We also used the two-hybrid system to identify the part of Sec16p that interacts with the C-terminal domain of Sec24p. The C terminus of Sec24p (amino acids 666–926) fused to an acidic activation domain was tested for interaction with three Sec16p fragments fused to the *lexA* DNA-binding domain (Figure 1B). The C-terminal domain of Sec24p interacted only with the central domain of Sec16p (Figure 1B). Sec23p, in contrast, interacted only with the C-terminal domain

of Sec16p, but not the central domain (Figure 1B). These results show that Sec23p and the C-terminal domain of Sec24p bind to different regions of Sec16p.

Sec24p Binds to the Central Domain of Sec16p in Yeast Extracts

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

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

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

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

To reconstitute the binding of Sec24p and Sec23p to Sec16p in vitro, we required a source of pure Sec23p and Sec24p. Sec23p and Sec24p were expressed as GST-fusion proteins in *E. coli*, purified by their affinity to glutathione beads, and then released from the beads

Figure 2. Affinity isolation of complexes among Sec24p, Sec16p, and Sec23p from yeast extracts. The indicated fragments of Sec24p fused to GST (lanes 1–6) or GST only (lanes 7 and 8) were coexpressed with either the central domain of Sec16p fused to invertase (pRH317, top panel) or Sec23p (pPE123, bottom panel). GST-fusion and associated proteins were purified by affinity to glutathione-agarose. Proteins were detected by Western blotting after SDS-PAGE on a 6% gel. Lanes 1, 3, 5, and 7 show 100,000 $\times$ g supernatant from 0.044 OD₆₀₀ U of cells. Lanes 2, 4, 6, and 8 show material bound to beads from 0.73 OD₆₀₀ U of cells.

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

by cleavage with thrombin (see MATERIALS AND METHODS). Sec23p purified in this manner was >90% pure, as judged by Coomassie-stained SDS-PAGE (Figure 3A, lane 1). Optimal expression of Sec24p required induction at low temperature in a multiple protease-deficient strain (*lon⁻ ompT⁻*) to prevent proteolytic degradation. After purification, the Sec24p preparation contained two major contaminating proteins (Figure 3A, lane 2); these proteins are probably bacterial proteins that bind nonspecifically to glutathione beads, because they were also present at much lower levels in the Sec23p preparations and did not react with anti-Sec24p antibodies. We used this partially purified preparation of Sec24p for all of the experiments described below.

Bacterially expressed Sec24p and Sec23p were tested for their ability to bind to each other in vitro (Figure 3B). Beads containing purified GST-Sec23p coprecipitated all of the Sec24p added (Figure 3B, lanes 3 and 4), whereas no binding of Sec24p was detected to glutathione beads alone (Figure 3B, lanes 5 and 6). Using the ability to bind to Sec23p as a criterion for function, we found that all of the recombinant Sec24p seemed to be active. The ability of recombinant Sec23p to bind to recombinant Sec24p in solution confirmed that no additional yeast proteins were required for association of Sec23p with Sec24p, as had been suggested previously (Hicke *et al.*, 1992).

Binding of Sec23p and Sec24p to Sec16p In Vitro

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

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

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

Sec23p and Sec24p copurify as a 300–400 kDa complex from yeast cytosol (Hicke *et al.*, 1992). To test whether Sec23p and Sec24p can form a complex when bound to Sec16p, we added both Sec23p and Sec24p to GST fused to the central domain of Sec16p. If Sec24p can bind simultaneously to the central domain of Sec16p and to Sec23p, Sec24p should recruit Sec23p to the GST-Sec16p fusion protein. Importantly, these experiments used a Sec16p fusion to which Sec23p alone would not bind. In the presence of Sec24p, a significant portion of Sec23p bound to the central domain of Sec16p (compare Figure 4C, lanes 3 and 4, with Figure 4B, lanes 1 and 2). Furthermore, the presence of Sec23p

Figure 4. Sec24p binds to the central domain of Sec16p in vitro. The binding reactions in A and B contain one of the following GST-fusion proteins immobilized on glutathione agarose: lanes 1–3, 11 pmol of GST-Sec16p_(565–1235); lanes 4–6, 13 pmol of GST-Sec16p_(1638–2194); lanes 7–9, 11 pmol of GST only. To test binding, we incubated the immobilized proteins with 2 pmol of recombinant Sec24p (A), 2 pmol of recombinant Sec23p (B), or buffer for 1 h at 22°C. Bound (B) and soluble (S) fractions were resolved by SDS-PAGE on 10% gels, and proteins were detected by Western blotting with anti-Sec24p (A, top panel), anti-Sec23p (B, top panel) or anti-GST antibodies (bottom panels). (C) GST-Sec16p_(565–1235) (11 pmol) was incubated with 2 pmol of recombinant Sec24p (lanes 1–4) and either 3 pmol of recombinant Sec23p (lanes 3 and 4) or buffer (lanes 1 and 2) for 1 h at 22°C. Bound (B) and soluble (S) fractions were recovered as above. Proteins were detected by Western blotting after SDS-PAGE on a 10% gel. (D) GST-Sec16p_(565–1235) (11 pmol) was incubated with 2 pmol of recombinant Sec24p (lanes 1–4) and the indicated amounts of recombinant Sec23p for 1 h at 22°C. Bound (B) and soluble (S) fractions were recovered as above. Sec24p was detected by Western blotting after SDS-PAGE on a 8% gel. Blots were quantitated by densitometry, and the average from two independent experiments is shown. Percentage bound = B/B + S. (E) The GST-fusion proteins affinity-purified from yeast that were used in the binding reactions were separated by SDS-PAGE on a 10% gel and stained with Coomassie brilliant blue: lane 1, 17 pmol of GST-Sec16p_(565–1235); lane 2, 20 pmol of GST-Sec16p_(1638–2194); lane 3, 17 pmol of GST.

significantly increased the amount of Sec24p bound to the central domain of Sec16p (Figure 4C, compare lanes 1 and 2 with lanes 3 and 4). Optimal binding of Sec24p to the central domain of Sec16p required stoichiometric amounts of Sec23p (Figure 4D). These data argue that Sec24p has distinct binding sites for the central domain of Sec16p and for Sec23p and show

that Sec23p and Sec24p can bind to Sec16p as a complex.

To evaluate the relative strengths of these protein-protein interactions, we examined the stability of binding at different concentrations of NaCl (Figure 5). Binding of Sec24p to the central domain of Sec16p was disrupted by 150 mM NaCl (Figure 5A), whereas bind-

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

ing of Sec23p to the C-terminal of Sec16p was stable up to 300 mM NaCl (Figure 5C). The presence of Sec23p did not affect the salt sensitivity of the interaction between Sec24p and the central domain of Sec16p (Figure 5B). These data suggest that Sec23p binds more tightly to Sec16p than Sec24p and argue that the stability of the interaction between Sec24p and the central domain of Sec16p is unchanged by Sec23p.

DISCUSSION

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

Because Sec24p bound to the central domain of Sec16p could also recruit Sec23p to bind to this region of Sec16p, we suggest that Sec23p and Sec24p can bind to Sec16p in a ternary complex. It is possible that Sec23p and Sec24p are always in a complex and that the proteins bind to Sec16p as a unit. Alternatively, Sec23p and Sec24p may bind to Sec16p in sequential steps of coat assembly to build a Sec23p/Sec24p/Sec16p complex. Interestingly, Sec23p seems to positively regulate the interaction between Sec24p and Sec16p, because the presence of Sec23p increases the amount of Sec24p that will bind to Sec16p *in vitro*. This increased binding could simply reflect the stoichiometry of Sec24p binding to Sec16p. For example, if the Sec23p/Sec24p complex contains two copies of Sec24p, formation of a Sec23p/Sec24p complex should double the number Sec24p molecules that can bind to each molecule of Sec16p. Alternatively, increased binding could result from a conformational change in Sec24p induced by Sec23p that increases the affinity of Sec24p for Sec16p. The Sec23p/Sec24p/Sec16p complex showed similar sensitivity to 150 mM salt as did the Sec24p/Sec16p complex, suggesting that Sec23p does not greatly increase the strength of the interaction between Sec24p and Sec16p.

Because Sec16p, Sec23p, and Sec24p are all components of the COPII vesicle coat, the protein–protein interactions that we have detected are likely to reflect

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

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

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

and the template onto which they assemble, will allow us to test this hypothesis.

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