COPII Subunit Interactions in the Assembly of the Vesicle Coat

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In vitro analysis of COPII vesicle formation in the yeast Saccharomyces 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 nonpermissive temperatures. We propose that Sec16p organizes the assembly of a coat that is stabilized both by the interaction of Sec31p with Sec23p and Sec24p and by the interaction of these three components with Sec16p.

In eukaryotic cells, proteins in the secretory pathway are transported from the endoplasmic reticulum (ER) to the Golgi apparatus via membrane-bounded vesicles (1) that are formed by the recruitment and assembly of cytosolic coat components upon the ER membrane (1–3). In the yeast Saccharomyces cerevisiae, three cytoplasmic factors, collectively termed COPII, have been shown to be required for vesicle formation: COPII subunits Sec23p and Sec24p (6, 12). We now report that Sec16p, an essential 240-kDa 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); Sec16p cannot be extracted from membranes by urea (6). Membranes prepared from sec16 mutant strains exhibit a marked deficit in the release of vesicle cargo molecules (11). Finally, Sec16p directly binds the COPII subunits Sec23p and Sec24p (6, 12). We now report that Sec16p also binds the COPII subunit Sec31p; we show this interaction is required for ER to Golgi transport.

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; B&H); anti-Sec23 (1/250) (14); anti-Sec24p (1/250) (14); anti-Sec13p (1/250) (15); and anti-Sec31p (1/100) (5). The antibodies against Sec16p, Sec23p, and Sec31p were generously provided by R. Schekman. LexA fusion constructs were made in pBTM116 and represent the entire coding sequence of yeast Sec13p (pDS135) (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 that utilizes the GAL1 promoter instead of the ADH1 promoter; the vector backbone of pGilda is from pRS313 (24). The strain CKY556, which is EGY40 (17) transformed with the indicator plasmid pSH18-24 (18), 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 and then galactose was added to 2%, and growth continued for another 4 h. 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 × A420/unit reaction time × A3OD units assayed) (25). All values above background were within 35% of the mean value.

In Vitro Binding Studies—Recombinant Sec23p and Sec24p were expressed as GST fusion proteins in Escherichia coli, purified by affinity chromatography, and then released by thrombin cleavage of the GST moiety (12). Recombinant Sec13p (kindly provided by K. Saxena and E. Neer) was purified as a His6 fusion protein from E. coli (26). The HA epitope-tagged Sec16 protein was prepared by insertion of a fragment encoding amino acids 447–1043 of SEC16 into the pGAL10-HA expression vector pRH165 to generate pDS216. This plasmid was transformed into the S. cerevisiae strain CKY557 (MATa ura3-52 trpl::hisG GAL1). Cells were grown to exponential phase in selective medium containing 2% raffinose and then supplemented with 2% galactose for 4–6 h 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, 0.1 mM NaCl) supplemented with protease inhibitors phenylmethylsulfonyl fluoride (1 mM),

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

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leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml) as well as EDTA (0.5 mM) and 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 centrifugation at 3,000 × g for 5 min, followed by centrifugation at 100,000 × g for 40 min.

DNA regions encoding the entire Sec1p 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 GST coding sequence expressed from pGAL1 promoter in pPE127, a vector identical to pDR56 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 Biotech Inc.) were incubated with the extracts for 30 min at 25 °C and 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 bovine serum albumin). 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, whereas binding of Sec13p (3 pmol) was carried out in 150 mM NaCl. After incubation for 1 h at 25 °C, the beads were washed twice or three times with binding buffer (no additional salt) and then resuspended in 0.25 mg/ml bovine serum albumin. The beads were then resuspended in extract sample buffer. All proteins were subjected to SDS-PAGE and Western blotting.

In Vivo Complementation Studies—Both the SEC31 deletion strain RSY1109 (MATa ade2-1 ura3-l leu2-3, 112 his3-11, 15 trp1-Δ1 s31::TRP1 [pRS811-SEC31-URA3-CEN]), kindly provided by R. Schekman, and the temperature-sensitive sec31-3 strain C135555 (MATa ura3-52 ade2-1 leu2-3, 112) kindly provided by A. Frank, were transformed with pDS21, pDS27, pDS28, or pDS415 (Strategene). sec31-2 was identified by A. Frank 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). pDS21 contains the full SEC31 genomic locus (6.2-kilobase BumHI-PstI fragment) inserted into the CEN, LEU2-marked pRS415 vector, pDS27, and pDS28 both contain a 5.0-kilobase SalI/SalI genomic SEC31 fragment, which represents a truncation that removes the coding sequence for the C-terminal 98 amino acids of the protein. pDS27 is a CEN-based plasmid derived from pRS415, and pDS28 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) and then shifted to nonpermissive temperature (36 °C) for 20 min. Pulse labeling of cells and immunoprecipitation of CPY were performed as described previously (23).

RESULTS AND DISCUSSION

Portions of the SEC16 coding sequence were surveyed for regions that would not by themselves act as transcriptional 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 SEC16 inserted into the pBTM116 LexA fusion vector (19), was one of the constructs that fulfilled this criterion. This fragment of Sec16p included the region known to bind Sec24p (Sec16p amino acids 565–1235) and was different from the region known to bind Sec23p (Sec16p amino acids 1638–2194) (6). The L444 receptor 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 8 × 105 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

![FIG. 1. Dissection of Sec31p binding domains through the use of two-hybrid analysis. A cDNA 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). A series of Sec31p truncations fused to an activation domain (pDS131 (1–490), pDS134 (570–1175), and pDS135 (850–1175)) were evaluated against lexA fusions to SEC13, SEC24, and SEC16 (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-Sec31p fusion protein with the GAL1-regulated vector pGilda. The regions of Sec31p that contain WD-40 repeats and proline-rich sequences are indicated in the diagram.](image-url)
yeast. This approach has been used previously to demonstrate the direct binding of Sec23 and Sec24p to different regions of Sec16p (12). 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). For the binding experiments, purified recombinant Sec23p, Sec24p, and Sec13p, or cytosol from yeast expressing amino acids 447–1043 of Sec16p tagged with the HA epitope. The results from these binding experiments were in complete agreement with the two-hybrid data (Fig. 2). As a control for protein expression levels, the wild-type yeast strain CYK8 (6) was transformed with either a CEN plasmid or Sec31p −null strain bearing wild-type SEC31 or sec31−ΔC. Transformants were grown with selection for the URA3-carbon plasmid, or with vector alone. Extracts from both sec31−ΔC and LEU2 selection for the sec31−null strain bearing wild-type SEC31 or sec31−ΔC. Transformants were grown with selection for the URA3-carbon plasmid, or with vector alone. Extracts from both sec31−ΔC and LEU2 plasmids carry- mers were plated on medium containing 0.1% 5-FOA to assay for complementation of the null allele. B. CKY555 transformants were plated at both permissive (24 °C) and nonpermissive (36 °C) temperatures to assay complementation of the sec31-2 allele. C, the transport of the Sec13p binding, whereas Sec31ΔCp was specifically defective for Sec16p binding. Sec23p and Sec24p bound to both of the truncated proteins, but not to GST alone; these binding reactions were performed in buffer containing 50 mM NaCl because very little binding of Sec24p and only about half-maximal binding of Sec23p were observed under the higher salt concentrations (150–200 mM NaCl) used for the Sec16p and Sec13p binding experiments (data not shown).

The identification of Sec31ΔCp, which is specifically defec-

tive 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 CYK8 (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 produc-

COPII Subunit Interactions

**Fig. 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 glutathione-agarose and incubated with recombinant Sec23p, Sec24p, Sec13p, or cytosol from yeast expressing amino acids 447–1043 of Sec16p tagged with the HA epitope. Samples representing the total protein added and the fraction bound were resolved by SDS-PAGE and Western blotting. The loading ratio of total to bound is 1:1 for the three recombinant proteins, and 1:10 for the recombinant Sec23p, Sec24p, and Sec13p were used. The source of Sec16p for this experiment was clarified extracts of yeast over-

**Fig. 3. Sec16p-binding region of Sec31p is essential and required for ER to Golgi transport.** Plasmids expressing either Sec31p (CEN plasmid) or Sec31ΔC (both CEN and 2µ plasmids) were trans-
formers were plated on medium containing 0.1% 5-FOA to assay for complementation of the null allele. B. CKY555 transformants were plated at both permissive (24 °C) and nonpermissive (36 °C) temperatures to assay complementation of the sec31-2 allele. C, the transport of the Sec16p-binding region of Sec31p is essential and required for ER to Golgi transport. Plasmids expressing either Sec31p (CEN plasmid) or Sec31ΔC (both CEN and 2µ plasmids) were trans-
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observed at nonpermissive 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 nonpermissive temperature of 36 °C was then evaluated. Although mutants transformed with the SEC31 plasmid grew at 36 °C, mutants transformed with the sec31ΔCp plasmid remained temperature-sensitive for growth (Fig. 3B); these results were observed in mutants transformed with either a CEN-based or a 2μ-based sec31ΔC plasmid. The kinetics of ER to Golgi transport of the marker cargo protein CPY was followed by pulse-chase analysis of the transformants at 36 °C. The sec31ΔC 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 that 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 complex, consisting of seven subunits that 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 detected between purified components of the Sec31p-Sec13p complex and the Sec23p-Sec24p complex suggested that these two complexes might preassemble in the cytosol. To examine this possibility, we expressed a GST-Sec31p fusion protein in yeast and asked whether Sec23p or Sec24p 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 was consistent with our measurements of the stability of the interactions between isolated proteins: binding of GST-Sec31p to recombinant Sec23p and Sec24p was demonstrated at 50 mM NaCl but (as noted above) was significantly weaker at 150 mM NaCl, a salt concentration equivalent to that used for the in vitro assay (data not shown). Thus, it is likely that the physiological association between the two COPII complexes requires the context of the ER membrane.

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 and Sec24p, we propose that Sec16p functions as a foundation for the construction of the COPII coat from soluble protein complexes (Fig. 4). Moreover, 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.

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FIG. 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 (12). 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. The direct interaction of Sar1p with Sec16p has not been demonstrated.