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Sec16p potentiates the action of COPII proteins to bud transport vesicles

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SEC16 encodes a 240-kD hydrophilic protein that is required for transport vesicle budding from the ER in *Saccharomyces cerevisiae*. Sec16p is tightly and peripherally bound to ER membranes, hence it is not one of the cytosolic proteins required to reconstitute transport vesicle budding in a cell-free reaction. However, Sec16p is removed from the membrane by salt washes, and using such membranes we have reconstituted a vesicle budding reaction dependent on the addition of COPII proteins and pure Sec16p. Although COPII vesicle budding is promoted by GTP or a nonhydrolyzable analogue, guanylimide diphosphate (GMP-PNP), Sec16p stimulation is dependent on GTP in the reaction. Details of coat protein assembly and Sec16p-stimulated vesicle budding were explored with synthetic liposomes composed of a mixture of lipids, including acidic phospholipids (major–minor mix), or a simple binary mixture of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Sec16p binds to major–minor mix liposomes and facilitates the recruitment of COPII proteins and vesicle budding in a reaction that is stimulated by Sar1p and GMP-PNP. Thin-section electron microscopy confirms a stimulation of budding profiles produced by incubation of liposomes with COPII and Sec16p. Whereas acidic phospholipids in the major–minor mix are required to recruit pure Sec16p to liposomes, PC/PE liposomes bind Sar1p-GTP, which stimulates the association of Sec16p and Sec23/24p. We propose that Sec16p nucleates a Sar1-GTP–dependent initiation of COPII assembly and serves to stabilize the coat to premature disassembly after Sar1p hydrolyzes GTP.

Introduction

The transport of proteins between secretory organelles is mediated by coated vesicles that capture cargo, bud from the donor membrane, and then deliver their content by fusing with a recipient organelle. Vesicles budding from the ER are coated with the COPII proteins, which comprise two multimeric complexes (Sec23/24p and Sec13/31p), and Sar1p, a GTPase of the Ras superfamily (Barlowe et al., 1994). COPII-coated vesicle assembly is initiated by the exchange of GDP for GTP on Sar1p, producing an activated form of Sar1p that embeds in the ER membrane and serves to recruit coat subunits (Matsuoka et al., 1998). This process is accompanied by coat polymerization, which shapes the forming bud and simultaneously incorporates cargo proteins and targeting machinery into the budding vesicle (Springer et al., 1999). Finally, coat disassembly is initiated by GTP hydrolysis, destabilizing the interactions that link Sar1p to the other coat proteins and to the membrane (Antonny et al., 2001). Other coat proteins, such as COPI and clathrin, employ similar mechanisms of assembly and disassembly (Rothman and Wieland, 1996; Schmid, 1997; Bremser et al., 1999).

The formation and consumption of activated Sar1p regulates the localization and lifetime of the COPII coat. Sec12p, an integral ER membrane glycoprotein, catalyzes nucleotide exchange on Sar1p, leading to the deposition of the activated protein in its proper location (Barlowe and Schekman, 1993; Sato et al., 1996). Coat assembly proceeds with the recruitment of Sec23/24p and then Sec13/31p. Coat disassembly, which is essential to the targeting of vesicles to the intermediate compartment, is intimately linked to two features of coat subunits: Sec23p, which is a Sar1p GTPase-activating protein (GAP),* and Sec13/31p, which stimulates Sec23

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Key words: COPII; Sar1p; Sec16p; vesicle budding; liposomes

*Abbreviations used in this paper: DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; GAP, GTPase-activating protein; GMP-PNP, guanylimide diphosphate; gpαF, glycopro-α-factor; MBP, maltose-binding protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIC, protease inhibitor cocktail.

GAP activity in the context of coat polymerization (Yoshihisa et al., 1993; Antonny et al., 2001). Similarly, the COPI coat is regulated by the GTP-binding protein Arf and localized nucleotide exchange catalysts, Sec7 domain–containing proteins, and Arf GAPs, which act in cooperation with the coat assembly protomer, coatomer (Cukierman et al., 1995; Chardin et al., 1996; Goldberg, 1999).

The interplay of Arf GTPase regulatory elements has been proposed to mediate the selectivity of cargo protein sorting into COPI vesicles (Lanoix et al., 1999; Goldberg, 2000). Although the efficiency of cargo sorting by COPII appears not to be diminished in incubations containing the nonhydrolyzable analogue guanylimide diphosphate (GMP-PNP), negative regulation of GTP hydrolysis may be important to stabilize the assembled coat during cargo capture in a forming bud. However, no such negative regulator of Sar1p GTPase has been detected.

In the absence of any such regulatory influence, COPII-mediated vesicle budding from synthetic phospholipid liposomes is sustained in reactions containing GMP-PNP, but not GTP (Matsuoka et al., 1998). The acceleration of GTP hydrolysis produced by COPII assembly on liposomes may be incompatible with the completion of fission of coated buds from a donor liposome. Thus, some protein factor, possibly cargo molecules or v-SNAREs, may serve to regulate Sar1p GTPase or otherwise stabilize the COPII coat to promote the GTP-dependent budding of COPII vesicles from ER membranes.

An obvious candidate for such a regulatory role is Sec16p. Extensive genetic evidence points to an essential role for *SEC16* in the generation of transport vesicles from the ER (Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990; Gimeno et al., 1995). Molecular cloning and localization analysis showed that Sec16p is a large, 240-kD, hydrophilic protein that associates peripherally with the ER membrane and contains domains that make direct contact with the COPII subunits Sec23p, Sec24p, and Sec31p (Espenshade et al., 1995; Gimeno et al., 1996; Shaywitz et al., 1997). Although Sec16p has been shown to participate in the production of COPII vesicles from ER membranes in vitro (Espenshade et al., 1995), it is not required for the budding reaction per se, because the COPII proteins suffice for the reaction reconstituted with liposomes.

In this paper, we describe the purification of a functional form of Sec16p. We used the pure protein to identify a role for Sec16p in the initiation of COPII coat assembly and to reconstitute a stimulation of vesicle formation in the liposome budding reaction. Our results suggest that Sec16p may stabilize a coat assembly intermediate without regulating Sar1p-GTP hydrolysis.

Results

Overproduction and purification of Sec16p

Sec16p is tightly and peripherally bound to the ER membrane (Espenshade et al., 1995). Therefore, as a starting material for Sec16p purification, we used a 10,000 *g* membrane fraction that was prepared from lysed yeast cells. Our goal was to solubilize Sec16p by treating the membranes with a buffer containing a high salt concentration and to purify the solubilized protein further by conventional chromatographic procedures. Because the amount of Sec16p present in the wild-type yeast cells was low, we placed *SEC16* under the control of the *GAL1* promoter in order to overproduce the protein. Induction with galactose resulted in an ~50-fold increase in the amount of membrane associated Sec16p. However, when membranes were treated with high salt, the solubilized Sec16p was almost completely degraded within 5 min (unpublished data). Although an extreme protease sensitivity of Sec16p was previously reported, the protein in a crude lysate was stabilized by a complex mixture of protease inhibitors (Espenshade et al., 1995). Unfortunately, a similar protease inhibitor cocktail was ineffective in stabilizing protein from overproducing cells. We replaced the *GAL1* promoter with a copper-inducible *CUP1* promoter and tested a few copper concentrations to induce different levels of Sec16p overproduction. Optimum overproduction and stability were achieved with cells grown to low density ($OD_{600} \leq 1.5$) and under the condition of constitutive induction with 0.4 mM $CuSO_4$, which

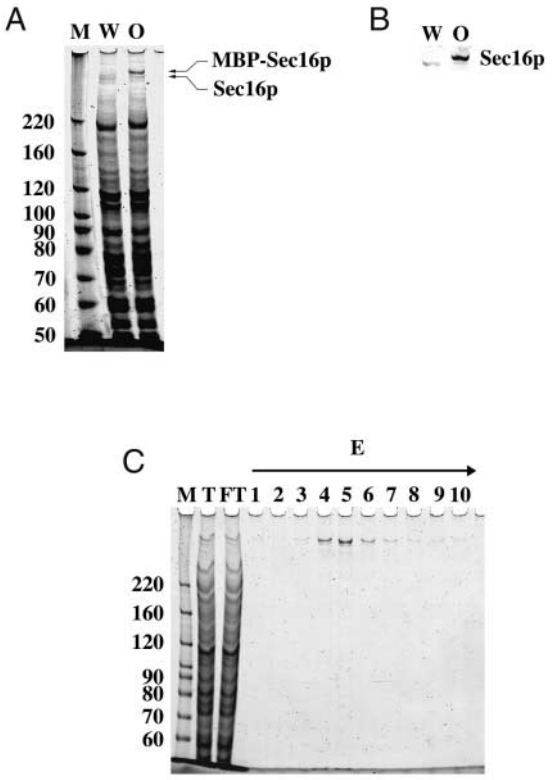

Figure 1. **Overexpression and purification of MBP–Sec16p.** (A) Protein composition of salt extracts from ER-enriched microsomes. 100 μg of microsomal membrane proteins from either wild-type FSY3 strain (W) or MBP–Sec16p-overproducing FSY9 strain (O) were incubated on ice in a 100-μl reaction containing 0.5 M NaCl for 15 min. After incubation, mixtures were centrifuged and 10 μl of supernatant fractions were separated on 6% SDS-PAGE and stained with SYPRO red. The left lane contains molecular weight standards (M). (B) Proteins were transferred to nitrocellulose and probed with anti-Sec16p antibody. (C) Salt extract from a 10,000 *g* membrane pellet was passed through a 6-ml amylose-agarose column and the bound protein was eluted with buffer containing 10 mM maltose. 10 1-ml fractions were collected. 2 μl of salt extract (T), flowthrough (FT), and fractions (E1–10) were separated on 6% SDS-PAGE and stained with SYPRO red stain.

does not result in growth inhibition by the overproduced Sec16p. These conditions allowed ~10-fold overproduction of Sec16p, which remained intact after it was eluted from broken membranes with high salt (Fig. 1, A and B).

Because a variety of purification procedures produced low yields (<10% at each of several chromatographic steps), we expressed Sec16p as a chimera containing an NH_2-terminal maltose-binding protein (MBP). The hybrid protein was functional based on its ability to support growth of a *sec16*-null mutant strain (unpublished data). MBP–Sec16p was readily detected as a major protein species on SYPRO red–stained SDS-PAGE of the salt-eluted fraction prepared from the overproducing cells (Fig. 1 A). The purification of fusion protein was achieved by applying a salt extract to an amylose column followed by elution with buffer containing 10 mM maltose. This single step yielded nearly homogeneous protein contaminated by low levels of Sec16p fragments (Fig. 1 C). A typical preparation yielded ~200 μg of protein from 700 g of yeast cells, with a variable but significant fraction of MBP–Sec16p lost in the amylose flowthrough.

Sec16p reconstitutes a GTP-dependent vesicle budding reaction

The formation of COPII-coated vesicles in vitro requires the ER membrane fraction, COPII proteins (Sar1p, Sec23/24p, and Sec13/31p), and a triphosphate form of guanine nucleotide (Barlowe et al., 1994). Under normal conditions of ER membrane isolation, Sec16p remains membrane bound and thus would not appear as a cytosolic requirement for vesicle budding. The harsh condition (3 M urea) used to remove residual COPII proteins from membranes (Salama et al., 1993) does not elute Sec16p (Espenshade et al., 1995). However, we found that Sec16p was quantitatively released into a supernatant fraction from membranes treated with 0.5 M NaCl (Fig. 2 A).

To develop a functional assay for soluble Sec16p, we characterized the packaging of [^{35}S]glycopro-α-factor ([^{35}S]gpαF) in COPII vesicle budding reactions containing salt-washed membranes. When the nonhydrolyzable GTP analogue GMP-PNP was used as a guanine nucleotide, only a small difference in budding efficiency was observed in reactions with or without MBP–Sec16p (Fig. 2 B). The maximum extent of budding at the saturating COPII concentration was similar in the presence or absence of MBP–Sec16p (Fig. 2 B). However, in incubations containing GTP, the absence of MBP–Sec16p resulted in less efficient budding, particularly in an incubation containing half the normal complement of COPII proteins. As the COPII concentration was increased, the defect was diminished, but not eliminated. We found similar results in vesicle budding reactions measuring the packaging of the SNARE protein Sec22p (unpublished data). These results suggest that Sec16p may influence the rate of GTP hydrolysis by Sar1p or enhance the stability of the COPII coat after GTP is hydrolyzed by Sar1p.

Sec16p facilitates the membrane association of COPII proteins

Liposomes reconstituted with pure phospholipids formulated to approximate the composition of a yeast microsomal membrane fraction support COPII coat assembly and vesicle budding (Matsuoka et al., 1998; Antonny et al., 2001). We examined the recruitment of Sec16p to such liposomes, referred to as major–minor mix, and the influence of Sec16p on the recruitment of COPII proteins. Sec16p efficiently bound to liposomes; ~20% of the input protein remained associated with liposomes floated up on a sucrose density gradient (Fig. 3 A). The presence of Sec16p stimulated the recruitment of Sec23/24p and Sec13/31p, but unlike the reaction stimulated by Sar1p and GNP-PNP (Fig. 3 A, lanes 1 and 2), the Sec16p-dependent reaction was Sar1p and nucleotide independent (Fig. 3 A, lane 6). Although the recruitment of Sec13/31p was inefficient (Fig. 3 A, lane 5), it was not dependent on prior assembly of Sec23/24p as it was in the Sar1p- and nucleotide-dependent reaction (Fig. 3 A, compare lanes 1 and 6). These results are consistent with two-hybrid and GST pull-down interaction experiments showing independent binding sites on Sec16p for Sec23p, Sec24p, and Sec31p (Espenshade et al., 1995; Gimeno et al., 1996; Shaywitz et al., 1997). In other experiments, we observed that a large proteolytic fragment of Sec31p (*species in Fig. 3 A, lane 1), missing a COOH-terminal domain

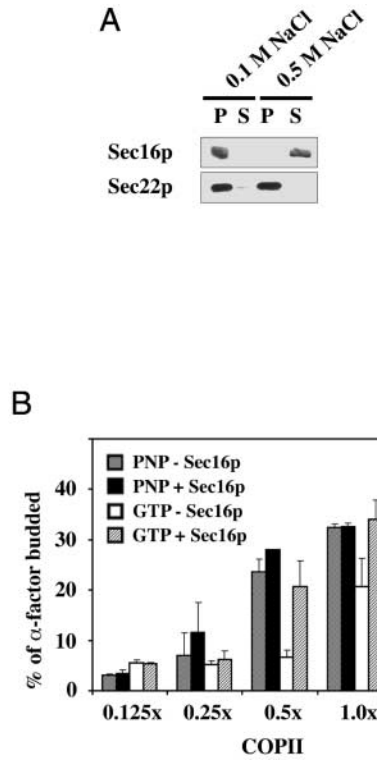

Figure 2. **Effect of Sec16p on vesicle budding.** (A) Microsomal membranes prepared from RSY267 were stripped as described in the legend to Fig. 1 with B88 buffer containing either 0.1 M NaCl or 0.5 M NaCl and centrifuged for 5 min at 10,000 *g*. Pellets were then resuspended in 100 μl of B88. 10 μl of both pellet (P) and supernatant fractions (S) were separated on 6% SDS-PAGE, transferred to nitrocellulose, and detected with the indicated antibody. (B) Vesicle release (% of total [^{35}S]gpαF released in vesicles) in the presence of saturating amounts of MBP–Sec16p (20 μg/ml) and various amounts of COPII proteins (standard conditions, 1 × COPII: 20 μg/ml Sar1p, 20 μg/ml Sec23/24p, and 50 μg/ml Sec13/31p). Membranes stripped with 0.5 M NaCl were used in budding reactions.

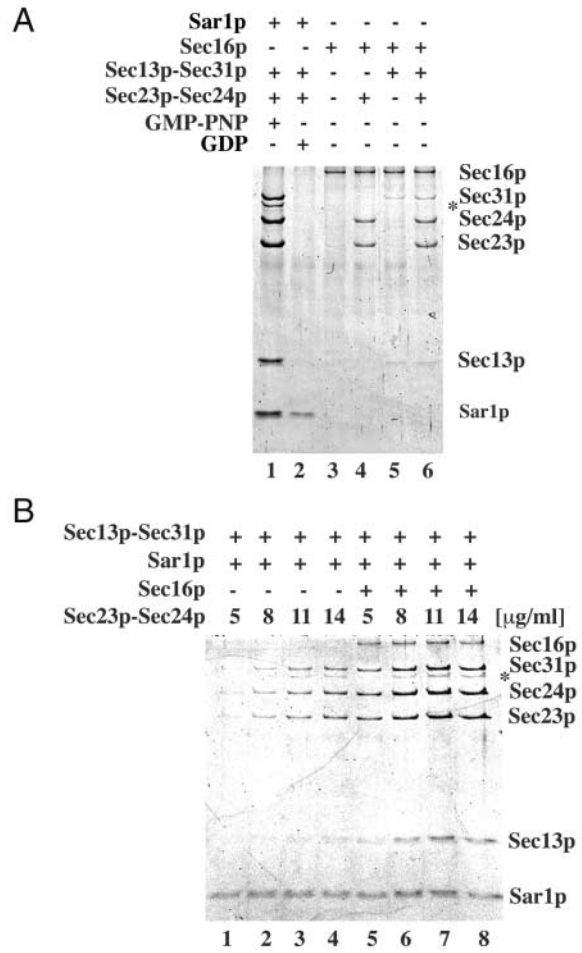

Figure 3. **Binding of MBP–Sec16p and COPII proteins to major–minor mix liposomes.** (A) Liposomes (corresponding to 25 μg of phospholipids) were incubated with indicated combinations of COPII proteins, MBP–Sec16p, and nucleotides (16 μg/ml Sar1p, 17 μg/ml Sec23/24p, 20 μg/ml Sec13/31p, 10 μg/ml MBP–Sec16p, and 0.1 mM GDP or GMP-PNP) for 15 min at 30°C in 250-μl reactions and then floated on top of a 0.7-M sucrose cushion. Equal amounts of lipids, measured using fluorescent phospholipids (Matsuoka et al., 1998), from floated fractions were applied to 11% SDS-PAGE and stained with SYPRO red. (B) Titration of Sec23/24p. The same amounts of Sar1p, Sec13/31p, MBP–Sec16p, and liposomes as in A were incubated with the indicated amounts of Sec23/24p in the presence of 0.1 mM GMP-PNP, and the binding of proteins was analyzed after liposome flotation. The asterisk indicates a truncated form of Sec31p.

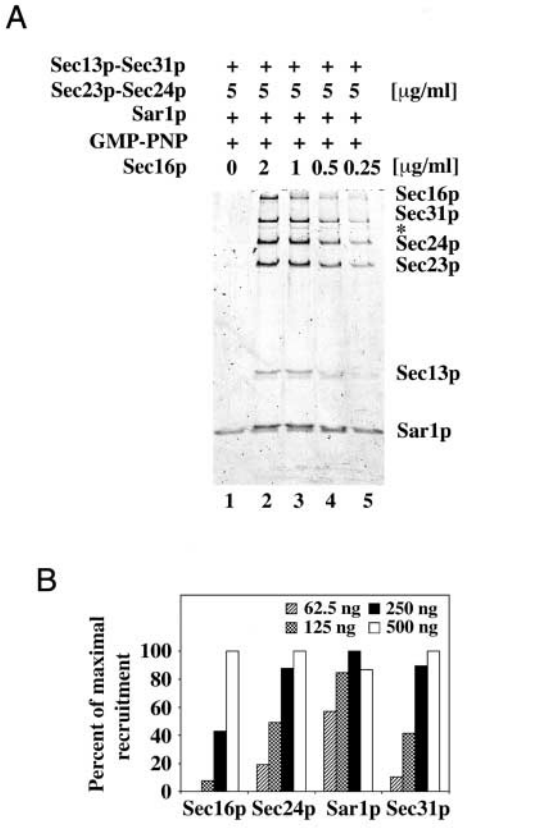

Figure 4. **Titration of MBP–Sec16p in a liposome binding reaction.** (A) Indicated concentrations of MBP–Sec16p were used for supplementation of binding reactions containing major–minor mix liposomes, COPII proteins (16 μg/ml Sar1p, 5 μg/ml Sec23/24p, and 20 μg/ml Sec13/31p), and GMP-PNP (0.1 mM). The asterisk indicates a truncated form of Sec31p. (B) Quantitation of bound proteins shown in A. The amounts of MBP–Sec16p present in 250-μl reactions are listed in the upper right corner of the graph.

known to be required for the interaction of Sec31p with Sec16p (Shaywitz et al., 1997), was not recruited to liposomes containing Sec16p with or without Sec23/24p. In contrast, this species was recruited in reactions containing Sar1p and nucleotide, probably because the truncated species retains a central domain responsible for interaction with Sec23/24p (Shaywitz et al., 1997), and this interaction may be potentiated by a Sar1p–GMP-PNP–Sec23/24p coat assembly complex.

We next examined the influence of Sec16p on the Sar1p–GMP-PNP–dependent recruitment of the COPII subunits. Sec23/24p was titrated in incubations with and without Sec16p (Fig. 3 B). In the presence of Sec16p at 0.5 μg/ml, an approximately threefold lower concentration of Sec23/24p was sufficient to recruit coat subunits (Fig. 3 B, compare lanes 4 and 5). In the absence of Sec16p, little, if any, COPII was assembled at the lowest concentration of Sec23/24p (Fig. 3 B, compare lanes 1 and 5). Optimum recruitment of COPII proteins was achieved in reactions containing 1 μg/ml of Sec16p (Fig. 4). These results demonstrate that Sec16p potentiates the nucleotide-dependent assembly of the COPII coat on an artificial membrane.

Matsuoka et al. (1998) showed that high concentrations of COPII proteins bud coated vesicles from liposomes (major–minor mix) incubated with GMP-PNP. Given the influence of Sec16p on the recruitment of COPII proteins to major–minor mix liposomes, we evaluated the contribution of Sec16p to the formation of COPII vesicles using a liposome budding assay in which small coated vesicles are resolved from donor membranes by sedimentation to equilibrium on a sucrose buoyant density gradient (Matsuoka et al., 1998). Fluorescently labeled liposomes incubated with saturating levels (5×; Matsuoka et al., 1998) of COPII proteins and GMP-PNP were loaded on sucrose gradients, centrifuged to equilibrium, and fractions were analyzed for lipid recovery. As reported previously (Matsuoka et al., 1998), two lipid peaks were observed (Fig. 5 A, top). The less dense material in fractions 6–8 is the result of partial COPII coating on

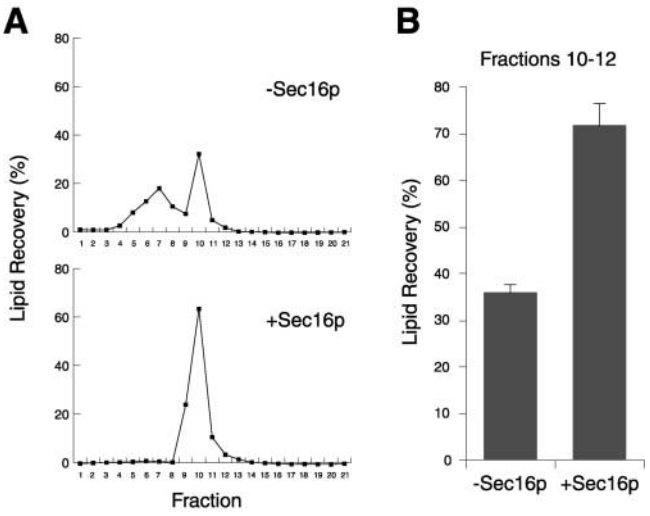

Figure 5. **Sec16p stimulates COPII vesicle formation from liposomes.** (A) Liposomes (corresponding to 12.5 μg phospholipids) were incubated with COPII proteins (80 μg/ml Sar1p, 130 μg/ml Sec23/24p, and 150 μg/ml Sec13/31p), MBP–Sec16p (11 μg/ml, where indicated), and GMP-PNP (100 μM) for 30 min at 27°C, and then sedimented to equilibrium on sucrose density gradients. The fluorescence of Texas red–labeled liposomes in each fraction was measured. (B) Average fluorescence of COPII vesicle peak (fractions 10–12) for three independent gradients (error bars are SEM).

large (400 nm) liposomes, whereas fractions 10–12 contain small COPII vesicles. When Sec16p was included in liposome budding reactions, the partially coated liposomes in fractions 6–8 were converted to COPII vesicles (fractions 10–12), resulting in a twofold stimulation of vesicle production (Fig. 5 B). Electron microscopic inspection of thin sections of unfractionated reactions conducted in the presence of Sec16p and saturating levels of COPII showed mainly small coated vesicles (unpublished data).

We also compared the morphology of liposomes incubated with or without Sec16p and nonsaturating levels of COPII (1×; Matsuoka et al., 1998) by thin-section electron microscopy. Membranes from reactions incubated at 30°C were floated on a sucrose step gradient, fixed, and processed for microscopy. As we reported previously, COPII buds and vesicles form on major–minor mix liposomes incubated with COPII proteins and GMP-PNP (Matsuoka et al., 1998; Fig. 6, A and B). Quantitation of this thin-section study revealed that in reactions with COPII only, 10.5% (0.7% error; $n = 1{,}137$) of all liposomes displayed vesicle profiles (buds and apparently separate vesicles of <90 nm in diameter), whereas 27.5% (1.4% error; $n = 1{,}029$) of membranes in incubations containing Sec16p displayed vesicle profiles. Addition of Sec16p also led to a pronounced morphological change in which buds and vesicles appeared clustered in the vicinity of large donor liposomes joined by coat protein material (Fig. 6 C).

GTP-dependent interaction of Sec16p and Sar1p

We observed a substantial influence of low concentrations of Sec16p on the recruitment of Sar1p–GMP-PNP to major–minor mix liposomes (Fig. 4, A and B), suggesting that the two proteins may directly interact. No molecular interaction had previously been described, however an indirect genetic interaction is known (Nakano and Muramatsu, 1989; Gimeno et al., 1995; Saito et al., 1999). To explore the possibility of a direct interaction, we examined the recruitment of Sec16p to liposomes formulated with phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (dioleoylphosphatidylcholine [DOPC]/dioleoylphosphatidylethanolamine [DOPE]), which we showed previously to bind Sar1-GTP or Sar1–GMP-PNP, but which fail to stably bind the other COPII proteins (Matsuoka et al., 1998).

We found that Sec16p did not bind PC/PE liposomes, but that Sar1p-GTP or Sar1p–GMP-PNP facilitated the recruitment of Sec16p (Fig. 7, lanes 1–4). Reactions containing Sec16p and Sar1p-GTP or Sar1–GMP-PNP supported the recruitment of Sec23/24p, but only weakly of Sec13/31p (Fig. 7, lanes 5, 6, 8, and 9), whereas the full set of COPII proteins bound only in the presence of Sec16p and Sar1–GMP-PNP (Fig. 7, lane 10). Surprisingly, the addition of Sec13/31p prevented the formation or accelerated the disassembly of a liposome–COPII subcomplex comprising Sec16p, Sar1p-GTP, and Sec23/24p (Fig. 7, compare lanes 5 and 7). These results suggest that Sec16p may help initiate the assembly of the COPII coat, preserving a liposome-bound complex including Sar1p-GTP and Sec23/24p. In the absence of Sec16p, this complex forms but is extremely unstable on major–minor mix and neutral liposomes, because the GAP activity of Sec23p converts Sar1p-GTP to Sar1p-GDP, leading to coat subunit dissociation from the liposome (Antonny et al., 2001; Fig. 8 B, lane 3).

The stability of the COPII subcomplex bound to PC/PE liposomes suggested that Sec16p may retard the Sec23p GAP or Sar1p GTPase activities, or that Sec16p may remain bound and tether Sec23/24p even after GTP hydrolysis by Sar1p. These possibilities were distinguished by direct Sar1p GTPase assays conducted in the presence of PC/PE liposomes and a limiting concentration of Sec23/24p with and without Sec16p. No effect of Sec16p on GTP hydrolysis was observed (Fig. 8 A), thus Sec16p is not a negative regulator of the GAP or GTPase activities. These results suggest an obligate sequence of events in which Sar1p-GTP recruits Sec16p followed by Sec23/24p. Subsequent GTP hydrolysis may not compromise the stability of this subcomplex, even though Sec16p, Sar1p, and Sec23/24p are not recruited to liposomes in the absence of GTP (Fig. 8 B, lane 10).

We next considered the possibility that Sec23/24p is recruited to the PC/PE liposome–COPII subcomplex in such a way as to interact with Sar1p-GTP and Sec16p simultaneously. Our standard incubation conditions could not distinguish one binding partner from another. As an alternative, we employed a novel inhibitor of Sar1p, BeF_x, which coordinates with GDP at the nucleotide binding site created by a complex of GTPase and GAP (Chabre, 1990; Scheffzek et al., 1998).Thus, unlike GTP and GMP-PNP, GDP and BeF_x will not activate a GTPase unless it is productively engaged in a complex with its cognate GAP. We previously used this technique to evaluate the interaction of Sar1p and Sec23/24p (Antonny et al., 2001).

When Sar1p, Sec23/24p, and nucleotide were incubated with PC/PE liposomes, only Sar1p bound (Fig. 8 B, lanes 1–3). Acidic phospholipids in the major–minor mix are re-

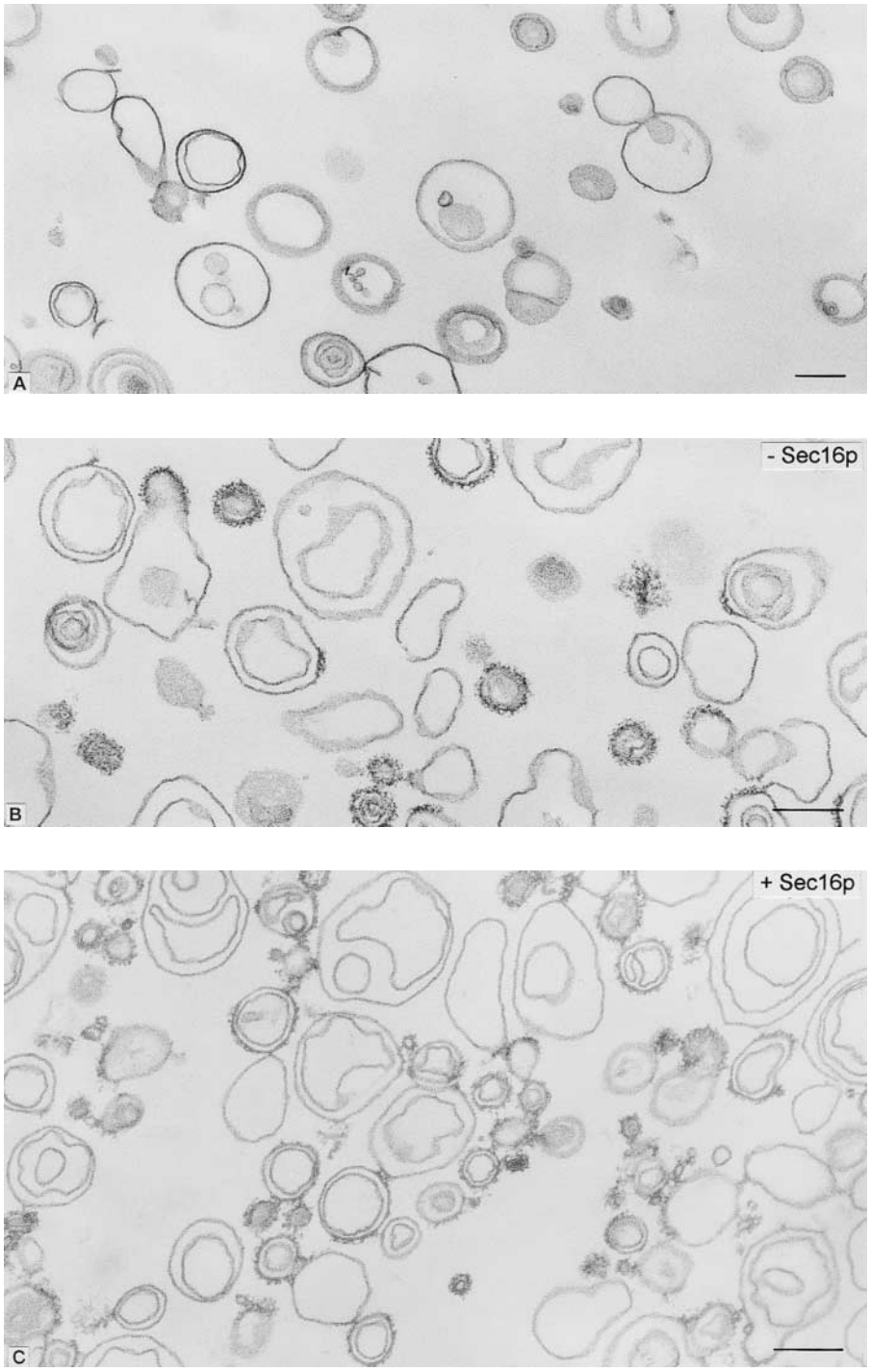

Figure 6. **Thin-section electron microscopy of major–minor mix liposomes incubated with and without COPII proteins, GMP-PNP, and MBP–Sec16p.** (A) No protein addition showing large, uncoated, uni- and multilamellar liposomes. (B) COPII and GMP-PNP promote coating, budding, and coated vesicle formation. (C) COPII, GMP-PNP, and Sec16p produce groups of vesicular profiles in close apposition to larger liposomes. Filamentous material not seen in B tethers liposomes and coated vesicles together. Bars, 0.2 μm.

quired to stably recruit Sec23/24p to a Sar1p–nucleotide–liposome complex (Matsuoka et al., 1998). Consequently, BeF_x and GDP were ineffective in assembling Sec23/24p into the complex (Fig. 8, lane 4), although this analogue is effective with major–minor mix liposomes (Antonny et al., 2001). Sec16p binding to PC/PE liposomes was Sar1p and GTP or GMP-PNP dependent (Fig. 7, lanes 3 and 4; Fig. 8 B, lanes 5–8), whereas BeF_x and GDP were ineffective (Fig. 8 B, lane 9). As before, Sar1p, Sec16p, and Sec23/24p were recruited to PC/PE liposomes in the presence of GTP or GMP-PNP (Fig. 7, lanes 5 and 8; Fig. 8 B, lanes 11–12). Finally, BeF_x and GDP facilitated the assembly of the liposome–COPII subcomplex at levels comparable to that achieved with GTP (Fig. 8 B, lane 13). Because GDP and BeF_x only activate GTPases complexed with GAP, we conclude that Sec23p must be catalytically engaged with Sar1p in the Sar1p-GTP–Sec23/24p–Sec16p subcomplex. Note that the level of Sar1p bound to liposomes was much lower in incubations that contained GTP or BeF_x and GDP than in an incubation with GMP-PNP (Fig. 8 B, compare lanes 11–13). Excess Sar1p-GTP not productively engaged in a COPII subcomplex may hydrolyze GTP and dissociate from vesicles, whereas Sar1p–GMP-PNP may remain vesicle bound outside of the context of a subcomplex. The low level of Sar1p-GDP/BeF_x observed

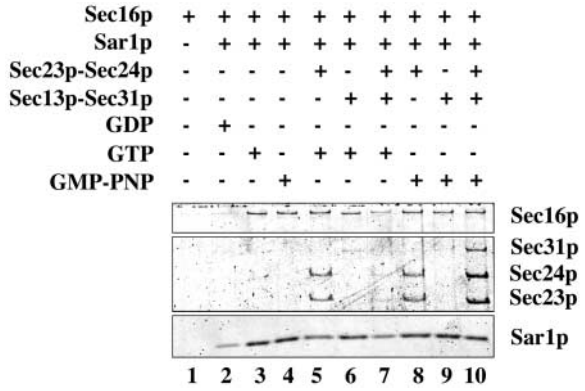

Figure 7. **Effect of MBP–Sec16p and GTP/GMP-PNP on COPII protein recruitment to DOPC/DOPE liposomes.** DOPC/DOPE liposomes (corresponding to 25 μg of phospholipids) were incubated with various combinations of COPII proteins, MBP–Sec16p, and nucleotides (48 μg/ml Sar1p, 17 μg/ml Sec23/24p, 20 μg/ml Sec13/31p, 10 μg/ml MBP–Sec16p, and 0.1 mM GDP, GTP, or GMP-PNP) for 15 min at 30°C in a 250-μl reaction. Proteins bound to liposomes were recovered by flotation, resolved on SDS-PAGE, and stained with SYPRO red.

is likely engaged exclusively in a subcomplex with Sec16p and Sec23/24p, just as Sar1p-GDP/BeF_x was found to be incorporated stoichiometrically to Sec23/24p on major–minor mix liposomes (Antonny et al., 2001).

Discussion

Sec16p is organized in domains and localized on the cytosolic surface of the ER in a manner that suggests a role in recruiting each of the proteins involved in forming a COPII coat. Kaiser's lab dissected Sec16p into domains that interact with three of the four proteins that comprise the bulk of the coat (Espenshade et al., 1995; Gimeno et al., 1996; Shaywitz et al., 1997). In addition, genetic evidence suggests a functional interaction between *SEC16* and Sar1p, the GTP-binding protein responsible for initiating coat assembly (Nakano and Muramatsu, 1989; Gimeno et al., 1995; Saito et al., 1999). However, the tight peripheral association of Sec16p with the ER membrane and the extreme proteolytic sensitivity of the protein have made a biochemical characterization of the role of the protein difficult. Until now, our studies relied on ER membranes that provide Sec16p, and on the cytosolic proteins required for transport vesicle budding, the subunits of COPII. By adjusting conditions of regulated induction of *SEC16* expression and by the use of an NH_2-terminal MBP chimera, we were able to develop a single step procedure to isolate pure, intact MBP–Sec16p. Nonetheless, the protein is not abundant and the yield of pure protein is low (~200 μg/700 g cells).

Membranes stripped of Sec16p respond to COPII proteins and bud transport vesicles containing gpαf and Sec22p nearly normally, provided the nonhydrolyzable GTP analogue, GMP-PNP, is included in the incubation. However, in the presence of GTP, a limiting concentration of COPII proteins produces a threefold stimulation by Sec16p. Previously, we demonstrated that Sar1p is shed and COPII subunits are less stably bound to vesicles formed in the presence of GTP, whereas the coat is retained on vesicles formed in the presence

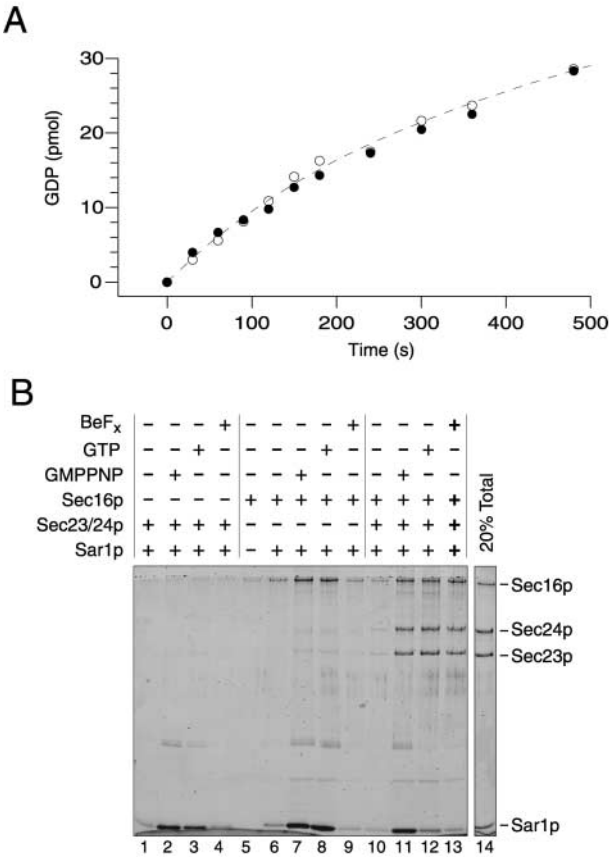

Figure 8. **Sec16p does not inhibit the GAP activity of Sec23p.** (A) GTPase assay. Sar1p-GDP (2 μM) was incubated with PC/PE liposomes and 10 μM [α-^{32}P]GTP for 10 min at 27°C to permit nucleotide exchange. Reactions were initiated by the addition of purified Sec23/24p to 105 nM in the presence (filled circles) or absence (open circles) of purified 50 nM Sec16p. (B) Flotation assay. Sar1p (15 μg/ml), Sec23/24p (20 μg/ml), and Sec16p (5 μg/ml) were incubated with PC/PE liposomes (200 μg/ml phospholipid) in the presence of GMP-PNP (100 μM), GTP (100 μM), or BeF_x (250 μM $BeCl_2$ and 10 mM KF) as indicated. Proteins bound to liposomes were recovered by flotation, resolved by SDS-PAGE, and stained with SYPRO red.

of GMP-PNP (Barlowe et al., 1994). A dynamic assay of coat assembly reveals a dramatic difference in coat stability in reactions containing GTP or GMP-PNP (Antonny et al., 2001). Thus, the effect of Sec16p implies some role in the coordination of GTP hydrolysis and vesicle budding or cargo protein capture into vesicles. Either Sec16p suppresses GTP hydrolysis by Sar1p during a crucial phase of cargo protein selection or completion of vesicle budding, or the COPII coat subunits are stabilized on the surface of a forming vesicle in contact with Sec16p after GTP hydrolysis is completed.

We evaluated the recruitment of COPII proteins by Sec16p using a liposome binding assay (Matsuoka et al., 1998). A liposome formulation containing a mixture of neutral and acidic phospholipids (major–minor mix) binds Sec16p in a Sar1p- and nucleoside triphosphate–independent process, which contrasts the recruitment of COPII proteins, which requires Sar1p and GTP or GMP-PNP (Antonny et al., 2001). Sec16p bound to major–minor mix liposomes recruits Sec23/24p or Sec13/31p without Sar1p

and nucleotide. However, the addition of Sec16p to a complete COPII incubation containing GMP-PNP facilitates the assembly of the coat and the formation of coated vesicles.

Although Sec16p facilitated coat assembly and vesicle budding in liposome reactions containing GMP-PNP, the reaction with native ER membranes was stimulated by Sec16p more dramatically in the presence of GTP. Unlike liposomes, the ER membrane is populated by membrane proteins that may assist in the recruitment of COPII subunits. We showed previously that Sar1p, GMP-PNP, and Sec23/24p form a complex with the cytosolic domain of SNARE proteins required to address COPII vesicles to the cis-Golgi (Springer and Schekman, 1998). This complex is not detected with GTP in place of GMP-PNP, probably because the SNAREs do not suppress the GAP activity of Sec23p and rapid GTP hydrolysis by Sar1p renders the complex unstable. Thus, if the principal role of Sec16p on the ER membrane is to stabilize COPII subunits, this function may be replaced by SNAREs or other proteins in incubations that contain GMP-PNP. However, Sec16p may play a unique and stimulatory role if it retains COPII proteins on the membrane after Sar1p hydrolyzes GTP.

To investigate this possibility, we exploited the observation that Sec16p binds neutral liposomes (PC/PE) only in the presence of Sar1p-GTP (or GMP-PNP). In contrast to the instability of a Sar1-GTP–Sec23/24p complex that forms on major–minor mix liposomes (Antonny et al., 2001), or on PC/PE liposomes (unpublished data), Sec16p stabilizes this association on PC/PE liposomes. This stabilization appears not to be the result of an inhibition by Sec16p of GTP hydrolysis by Sar1p and Sec23/24p. Furthermore, a productive complex including Sec16p forms in the presence of GDP and BeF_x, an analogue that complexes the GTP binding site of Sar1p only when it is bound to Sec23/24p (Antonny et al., 2001).

We propose that Sec16p organizes an initial step in the assembly of the COPII coat. Sec16p may be bound to the ER membrane in the vicinity of Sec12p, the nucleotide exchange catalyst of Sar1p (d'Enfert et al., 1991; Barlowe and Schekman, 1993). Sar1p-GTP would form and bind Sec16p, recruiting Sec23/24p to make a coat initiation complex. During the lifetime of Sar1p-GTP, cargo molecules destined for transport may engage the complex and be captured in advance of GTP hydrolysis (Kuehn et al., 1998; Springer et al., 1999). Although GTP hydrolysis is not required in protein sorting mediated by the COPII coat, it is an essential aspect of coat disassembly. Thus, GTP hydrolysis accelerated by the action of the Sec23 GAP on Sar1p (Yoshihisa et al., 1993), or further enhanced by the polymerization of the coat induced by Sec13/31p (Antonny et al., 2001), is required to produce an uncoated vesicle exposing SNARE proteins to a target membrane. Sec16p may act as a tether to prevent premature discharge of coat subunits before the completion of vesicle fission from the ER membrane.

Materials and methods

Strains and materials

Saccharomyces cerevisiae strain RSY267 (*MATα sec16–2 ura2–52 his4–619*) was used for the preparation of ER-enriched microsomes (Wuestehube and Schekman, 1992), and FSY3 strain (*Matα/Mata ade2–1/ADE2 can1–100/CAN1 his3–11,15/his3Δ200 leu2-Δ1/leu2–3,112 lys2–801/LYS2 prbΔ16R/PRB1 pep4::TRP1/pep4::HIS3 trp1-Δ1/trp1–1 ura3–1/ura3–52*) was used as a host for the overexpression of *SEC16*.

Most of the phospholipids and fluorescent phospholipid derivatives were purchased from Avanti Polar Lipids, Inc. Cytidinediphosphate diacylglycerol (CDP-DAG) and ergosterol were purchased from Sigma-Aldrich. Texas red DHPE and SYPRO red protein stain were purchased from Molecular Probes. ^{125}I-labeled protein A was purchased from ICN Biomedicals and the ECL PLUS Western Blotting System was purchased from Amersham Biosciences.

Published procedures were used to purify Sar1p, Sec23/24p (Barlowe et al., 1994), and Sec13/31p (Salama et al., 1993). Antibodies against Sec16p and Sec22p were described previously (Espenshade et al., 1995; Bednarek et al., 1995, respectively).

Construction of Sec16p overproducer

To facilitate purification of Sec16p, we fused the *Escherichia coli* malE gene to the NH_2-terminal coding sequence of *SEC16*, and the hybrid malE–*SEC16* gene was put under the control of the inducible *CUP1* promoter. The *CUP1*–malE–*SEC16* construct (pFS19) was assembled in pBluescript II SK+ vector (Stratagene). The *CUP1* promoter and the 5′ noncoding region of *SEC16* were amplified by PCR from yeast chromosomal DNA (W303-1B strain) using FS50 (CGAGTCCATTACCGACATTTGGGCGCTATAC) and FS51 (CGGATCCGTACAGTTTGTTTTTCTTAATATC) and FS52 (GATGATCACTTATAACGTTGTGTT) and FS53 (CGTCGACGAGCCATCTTTGTCAGGGTG) combinations of primers, respectively. PCR products were cloned into pCR2.1-TOPO vector (Invitrogen) and the identities of inserts in the resulting plasmids (p*CUP1* and p16N, respectively) were confirmed by sequencing. The *CUP1* promoter was then subcloned from p*CUP1* into the pBluescript II SK+ vector through BamHI and EcoRI restriction sites. The resulting plasmid, pBS-*CUP1*, was cleaved with XhoI and HindIII restriction enzymes and ligated with a SalI and HindIII p16N fragment containing the 5′ noncoding region of *SEC16*, yielding the p*CUP*-16N plasmid. Two more PCR reactions were performed in order to introduce convenient restriction sites at the ends of DNA fragments. The Kan^r gene from plasmid pUG6 (Guldener et al., 1996) and the malE gene from plasmid pMAL-c2 (New England Biolabs, Inc.) were amplified using FS54 (GAAGCTTCGTACGCTGCAGGTC) and FS55 (CGACACTGGATGGCGGCGTTAG) and FS62 (CGGATCCATGAAAACTGAAGAAGGTAAACTG) and FS63 (CGGATCCGCGTTGTTGTTCGAGCTCGAATTAG) combinations of primers, respectively. PCR products were cloned into pCR2.1-TOPO vector, sequenced, and the resulting plasmids were named pCR-malE and pCR-Kan, respectively. The pCR-Kan plasmid was cleaved with EcoRI and the fragment encoding the Kan^r gene was cloned into the EcoRI site of pCUP-16N to give pCUP-Kan plasmid. *SEC16* was fused to the *CUP1* promoter by isolating the BamHI-SacII DNA fragment (coding for the NH_2-terminal part of Sec16p) from pPE4 (Espenshade et al., 1995) and cloning it into BamHI-SacII sites in p*CUP*-Kan to give the pFS12 plasmid. In the last step, the malE gene from pCR-malE was introduced between the *CUP1* promoter and *SEC16* through a BamHI site, creating translational fusion of malE and *SEC16*. The final pFS19 plasmid contained the following genetic elements ordered from the 5′ end to the 3′ end: 5′ noncoding region of *SEC16*, Kan^r, *CUP1* promoter, malE, and the 5′ coding region of *SEC16*. The insert was excised from pFS19 with SacII and HindIII restriction endonucleases and was used for the transformation of diploid FSY3 strain. One transformant, FSY9, which contained the chromosomal replacement of one copy of the *SEC16* promoter with the *CUP1–malE* element, was selected for further experiments. The expression of the fusion protein was confirmed using antibodies directed against MBP (New England Biolabs, Inc.) and Sec16p.

Purification of Sec16p

A culture of FSY9 grown in YPD broth was used to inoculate SC medium supplemented with 0.1% (wt/vol) Casamino acids (Difco) and 0.4 mM $CuSO_4$. Cells were grown at 30°C in a 2001 New Brunswick fermenter and harvested at an OD_{600}/ml of 1.5. Cells were centrifuged, washed twice with distilled water, once with buffer JR (20 mM Hepes-KOH, pH 7.4, 50 mM KOAc, 2 mM EDTA, 0.2 M soribtol), and resuspended in a minimal volume of buffer JR. A typical yield from a 200-liter cultivation was ~700 g of wet yeast paste. Cells were frozen by slowly pouring the cell slurry into liquid N_2 and stored at −80°C. The frozen cell suspension was mixed with liquid nitrogen in a Waring blender to prepare a cell lysate. The blended lysate was thawed on ice and diluted with 1.5 liters of buffer JR containing 1 mM DTT and protease inhibitor cocktail (PIC; antipain, aprotinin, chymostatin, leupeptin, pepstatin, each at concentration of 1 μg/ml, 1 mM PMSF, and 0.1 mM TPCK). Unbroken cells and large membrane ag-

gregates were removed by centrifugation for 10 min at 500 *g* in a GS3 rotor (Sorvall; Dupont) and the resulting supernatant fraction was then further centrifuged for 30 min at 15,000 *g* in the same rotor. The pellet fraction from the second centrifugation was sequentially washed with 500 ml of buffer JR (supplemented with 1 mM DTT and PIC) and 500 ml of buffer B88 (20 mM Hepes-KOH, pH 6.8, 150 mM KOAc, 5 mM Mg $[OAc]_2$, 0.25 M sorbitol, 1 mM DTT) containing PIC. The resulting membrane fraction was resuspended in 200 ml of B88, frozen in liquid N_2, and stored at −80°C as 10-ml aliquots. To prepare a salt extract, the frozen membranes were slowly thawed on ice, washed with 250 ml of B88 and PIC, and resuspended in 200 ml of the same buffer. Peripheral membrane proteins were released into solution by adding 13 ml of 5 M NaCl (final, 0.3 M NaCl) to the membrane suspension. The mixture was rapidly stirred and incubated on ice for 15 min. The stripped peripheral proteins were separated from membranes by a 15-min centrifugation in the SS-34 rotor at 15,000 rpm. About 90% of MBP–Sec16p was released into the supernatant by this treatment. The supernatant was applied to a 6-ml amylose (New England Biolabs, Inc.) column equilibrated with B88 supplemented with PIC and 0.3 M NaCl, and the unbound proteins were removed by washing the column with 100 ml of B88 containing PIC and 0.5 M NaCl. Thereafter, the column was washed with 50 ml of B88 supplemented with PIC, 0.3 M NaCl, and 20% glycerol (vol/vol) and MBP–Sec16p was eluted with 10 ml of the same buffer containing 10 mM maltose. 10 1-ml fractions were collected and MBP–Sec16p elution was monitored by PAGE and SYPRO red staining. Three fractions containing the most concentrated MBP–Sec16p were pooled, aliquoted, frozen in liquid N_2, and stored at −80°C.

Vesicle budding reactions

To prepare salt-washed membranes, we thawed 1 mg of microsomal membranes (Wuestehube and Schekman, 1992) on ice, centrifuged the sample at 8,000 rpm for 5 min at 4°C in a microcentrifuge (Tomy Tech USA Inc.), and washed the membranes with 1 ml of ice-cold B88. The washed membranes were resuspended in 90 μl of B88, and 10 μl of 5 M NaCl was added. The mixture was rapidly vortexed and incubated on ice for 15 min. After centrifugation in a microcentrifuge at 15,000 rpm for 5 min at 4°C, the stripped membranes were washed two more times with 1 ml of B88 and resuspended in 100 μl of the same buffer. [^{35}S]prepro-α-factor was posttranslationally translocated into stripped microsomes as described previously (Latterich and Schekman, 1994). The translocation reaction was stopped by transferring it to ice and diluted with two volumes of B88 containing 1.5 M KOAc. After 15 min on ice, the membranes were centrifuged at 15,000 rpm, for 5 min at 4°C (Tomy Tech USA Inc.), washed twice with B88 buffer, and finally resuspended in 100 μl of the same buffer. 10 μl of stripped membranes were used in 50-μl budding reactions supplemented with the indicated concentrations of GTP/GMP-PNP, COPII proteins, and Sec16p. Reactions were incubated for 30 min at 30°C and the amount of gpαF packaged into vesicles was determined as previously described (Campbell and Schekman, 1997).

Liposome binding and budding assays

Liposome preparation, protein binding to liposomes, and vesicle budding from liposomes were performed as described previously (Matsuoka and Schekman 2000; Antonny et al., 2001). All liposomes used contained 80% (by weight) of phospholipids and 20% of ergosterol. The phospholipid component consisted of 49 mol% DOPC, 21 mol% DOPE, 8 mol% dioleoylphosphatidylserine (DOPS), 5 mol% dioleoylphosphatidic acid (DOPA), 8 mol% phosphatidylinositol (PI), 2.2 mol% PI-4-phosphate, 0.8 mol% PI-4,5-bisphosphate, 2 mol% cytidinediphosphate diacylglycerol (CDP-DAG), 2 mol% NBD-PE, and 2 mol% NBD-PC for major–minor mix liposomes and 51 mol% DOPC, 45 mol% DOPE, 2 mol% NBD-PC, and 2 mol% NBD-PE for DOPC/DOPE liposomes. Membranes were floated on a sucrose step gradient as described previously (Antonny et al., 2001). To analyze the protein content of floated liposomes, we applied equal quantities of lipids from the floated fractions onto SDS-PAGE and, after separation, proteins were stained with SYPRO red (Molecular Probes) and visualized using a STORM 860 image analyzer.

For liposome budding assays, 100-μl reactions containing buffer C (20 mM Hepes-KOH, pH 7.0, 290 mM KOAc, 15 mM sorbitol, 9% glycerol), major–minor mix liposomes (125 μg/ml phospholipids, labeled with 1 mol% Texas red DHPE), Sar1p (80 μg/ml), Sec23/24p (130 μg/ml), Sec13/31p (150 μg/ml), and MBP–Sec16p (11 μg/ml, where indicated) were incubated at 27°C for 30 min, loaded on a sucrose gradient (Matsuoka and Schekman 2000), topped with 100 μl B88, and centrifuged for 16 h in a TLS-55 rotor at 55,000 rpm at 4°C. Fractions (100 μl) were collected from the top of the gradient, and the fluorescence was measured in a microtiter plate using a Typhoon 9400 variable mode imager. The COPII vesicle peak (fractions 10–12) was displaced relative to the position previously reported (Matsuoka et al., 1998) because smaller samples were applied to the gradient.

For thin-section electron microscopy, we incubated reactions containing major–minor mix liposomes (330 μg/ml phospholipids) with and without Sar1p (15 μg/ml), Sec23/24p (20 μg/ml), Sec13/31p (30 μg/ml), and MBP–Sec16p (4 μg/ml) for 25 min at 27°C. Fractions (∼500 μl) were mixed with ∼300 μl 2.5 M sucrose and layered under 200 μl of 0.75 M sucrose both in buffer B (Antonny et al., 2001), and topped by 25 μl of buffer B. Samples were centrifuged at 55,000 rpm for 90 min at 4°C in a TLS-55 rotor of the TL100 ultracentrifuge (Beckman Coulter). The top 100 μl of each fraction was collected and processed for thin-section electron microscopy as described previously (Matsuoka et al., 1998).

GTPase assay

GTPase assays were performed as previously described, with modifications (Antonny et al., 2001). Sar1p-GDP (2 μM) was incubated in 75-μl reactions at 27°C for 10 min with or without 50 nM purified Sec16p and 10 μM GTP, 100 nCi [α-^{32}P]GTP, 100 μM ATP, and 300 μg/ml PC/PE liposomes. Hydrolysis was initiated by the addition of purified Sec23/24p to 105 nM.

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References

Antonny, B., D. Madden, S. Hamamoto, L. Orci, and R. Schekman. 2001. Dynamics of the COPII coat with GTP and stable analogues. *Nat. Cell Biol.* 3:531–537.

Barlowe, C., and R. Schekman. 1993. *SEC12* encodes a guanine nucleotide exchange factor essential for transport vesicle budding from the ER. *Nature.* 365:347–349.

Barlowe, C., L. Orci, T. Yeung, M. Hosobuchi, S. Hamamoto, N. Salama, M.F. Rexach, M. Ravazzola, M. Amherdt, and R. Schekman. 1994. COPII: a membrane coat formed by sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell.* 77:895–907.

Bednarek, S.Y., M. Ravazzola, M. Hosobuchi, M. Amherdt, A. Perrelet, R. Schekman, and L. Orci. 1995. COPI- and COPII-coated vesicles bud directly from the endoplasmic reticulum. *Cell.* 83:1183–1196.

Bremser, M., W. Nickel, M. Schweikert, M. Ravazzola, M. Amherdt, M. Hughes, T.H. Sollner, J.E. Rothman, and F.T. Wieland. 1999. Coupling of coat assembly and vesicle budding to packaging of putative cargo receptors. *Cell.* 96:495–506.

Campbell, J.L., and R. Schekman. 1997. Selective packaging of cargo molecules into endoplasmic reticulum-derived CopII vesicles. *Proc. Natl. Acad. Sci. USA.* 94:837–842.

Chabre, M. 1990. Aminofluoride and beryllofluoride complexes: a new phosphate analogs in enzymology. *Trends Biochem. Sci.* 15:6–10.

Chardin, P., S. Paris, B. Antonny, S. Robineau, S. Beraud-Dufour, C.L. Jackson, and M. Chabre. 1996. A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. *Nature.* 384:481–484.

Cukierman, E., I. Huber, M. Rotman, and D. Cassel. 1995. The ARF1 GTP-ase activating protein: zinc finger motif and Golgi complex localization. *Science.* 270:1999–2002.

d'Enfert, C., C. Barlowe, S. Nishikawa, A. Nakano, and R. Schekman. 1991. Structural and functional dissection of a membrane glycoprotein required for vesicle budding from the endoplasmic reticulum. *Mol. Cell. Biol.* 11: 5727–5734.

Espenshade, P., R.E. Gimeno, E. Holzmacher, P. Teung, and C.A. Kaiser. 1995. Yeast *SEC16* gene encodes a multidomain vesicle coat protein that interacts with Sec23p. *J. Cell Biol.* 131:311–324.

Gimeno, R.E., P. Espenshade, and C.A. Kaiser. 1995. *SED4* encodes a yeast endoplasmic reticulum protein that binds Sec16p and participates in vesicle for-

mation. *J. Cell Biol.* 131:325–338.

Gimeno, R.E., P. Espenshade, and C.A. Kaiser. 1996. COPII coat subunit interaction: Sec24p and Sec23p bind to adjacent regions of Sec16p. *Mol. Biol. Cell.* 7:1815–1823.

Goldberg, J. 1999. Structural and functional analysis of the ARF1-ARFGAP complex reveals a role for coatomer in GTP hydrolysis. *Cell.* 96:893–902.

Goldberg, J. 2000. Decoding of sorting signals by coatomer through a GTPase switch in the COPI coat complex. *Cell.* 100:671–679.

Guldener, U., S. Heck, T. Fielder, J. Beinhauer, and J.H. Hegeman. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* 24:2519–2524.

Kaiser, C.A., and R. Schekman. 1990. Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell.* 61:723–733.

Kuehn, M.J., J.M. Herrmann, and R. Schekman. 1998. COPII-cargo interactions direct protein sorting into ER-derived transport vesicles. *Nature.* 391:187–190.

Lanoix, J., J. Ouwendijk, C.C. Lin, A. Stark, H.D. Love, J. Ostermann, and T. Nilsson. 1999. GTP hydrolysis by arf-1 mediates sorting and concentration of Golgi resident enzymes into functional COPI vesicles. *EMBO J.* 18: 4935–4948.

Latterich, M., and R. Schekman. 1994. The karyogamy gene *KAR2* and novel proteins are required for ER-membrane fusion. *Cell.* 78:87–98.

Matsuoka, K., and R. Schekman. 2000. The use of liposomes to study COPII- and COPI-coated vesicle formation and membrane protein sorting. *Methods.* 20: 417–428.

Matsuoka, K., L. Orci, M. Amherdt, S.Y. Bednarek, S. Hamamoto, R. Schekman, and T. Yeung. 1998. COPII-coated vesicle formation reconstituted with purified coat proteins and chemically defined liposomes. *Cell.* 93:263–275.

Nakano, A., and M. Muramatsu. 1989. A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Biol.* 109:2677–2691.

Rothman, J.E., and F.T. Wieland. 1996. Protein sorting by transport vesicles. *Science.* 272:227–234.

Salama, N.R., T. Yeung, and R.W. Schekman. 1993. The Sec13p complex and reconstitution of vesicle budding from the ER with purified cytosolic proteins. *EMBO J.* 12:4073–4082.

Saito, Y., T. Yamanushi, T. Oka, and A. Nakano. 1999. Identification of *SEC12*, *SED4*, truncated *SEC16*, and *EKS1/HRD3* as multicopy suppressors of ts mutants of Sar1 GTPase. *J. Biochem.* 125:130–137.

Sato, M., K. Sato, and A. Nakano. 1996. Endoplasmic reticulum localization of Sec12p is achieved by two mechanisms: Rer1p-dependent retrieval that requires the transmembrane domain and Rer1p-independent retrieval that involves the cytoplasmic domain. *J. Cell Biol.* 134:279–293.

Scheffzek, K., M.R. Ahmadian, and A. Wittinghofer. 1998. GTPase-activating proteins: helping hands to complement an active site. *Trends Biochem. Sci.* 23:257–262.

Schmid, S.L. 1997. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.* 66:511–548.

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

Springer, S., and R. Schekman. 1998. Nucleation of COPII vesicular coat complex by endoplasmic reticulum to Golgi vesicle SNAREs. *Science.* 281:698–700.

Springer, S., A. Spang, and R. Schekman. 1999. A primer on vesicle budding. *Cell.* 97:145–148.

Wuestehube, L.J., and R.W. Schekman. 1992. Reconstitution and transport from endoplasmic reticulum to Golgi complex using endoplasmic reticulum-enriched membrane fraction from yeast. *Methods Enzymol.* 219:124–136.

Yoshihisa, T., C. Barlowe, and R. Schekman. 1993. Requirement for a GTPase-activating protein in vesicle budding from the endoplasmic reticulum. *Science.* 259:1466–1468.