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 Sec23p.

*Abbreviations used in this paper: DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; GAP, GTPase-activating protein; GMP-PNP, guanylimide diphosphate; gppF, glycopro-ß-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 lipid 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 (OD600 ≈ 1.5) and under the condition of constitutive induction with 0.4 mM CuSO4, which
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. 1A). 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 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. 1maltose. This single step yielded nearly homogeneous protein contaminated by low levels of Sec16p fragments (Fig. 1C). 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. 2A).

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. 2B). The maximum extent of budding at the saturating COPII concentration was similar in the presence or absence of MBP–Sec16p (Fig. 2B). 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. 3A). The presence of Sec16p stimulated the recruitment of Sec23/24p and Sec13/31p, but unlike the reaction stimulated by Sar1p and GNP-PNP (Fig. 3A, lanes 1 and 2), the Sec16p-dependent reaction was Sar1p and nucleotide independent (Fig. 3A, lane 6). Although the recruitment of Sec13/31p was inefficient (Fig. 3A, lane 5), it was not dependent on prior assembly of Sec23/24p as it was in the Sar1p- and nucleotide-dependent reaction (Fig. 3A, compare lanes 2 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. 3A, lane 1), missing a COOH-terminal domain

![Figure 2. Effect of Sec16p on vesicle budding.](Image 343x449 to 517x732)
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–GDP–PK Performs cocktail 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. 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
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 COPPI 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 COPPI 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–COPPI 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 COPPI 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 COPPI 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–COPPI 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-

large (400 nm) liposomes, whereas fractions 10–12 contain small COPPI vesicles. When Sec16p was included in liposome budding reactions, the partially coated liposomes in fractions 6–8 were converted to COPPI 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 COPPI showed mainly small coated vesicles (unpublished data).

We also compared the morphology of liposomes incubated with or without Sec16p and nonsaturating levels of COPPI (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, COPPI buds and vesicles form on major–minor mix liposomes incubated with COPPI proteins and GMP-PNP (Matsuoka et al., 1998; Fig. 6, A and B). Quantitation of this thin-section study revealed that in reactions with COPPI 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
quired to stably recruit Sec23/24p to a Sar1p–nucleotide–liposome complex (Matsuoka et al., 1998). Consequently, BeF₆ 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 BeF₆ 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, BeF₆ 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 BeF₆ 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 BeF₆ 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/BeF₆ observed...
Sec16p stimulates COPII budding

Supek et al. 1035

is likely engaged exclusively in a subcomplex with Sec16p and Sec23/24p, just as Sar1p-GDP/BeF<sub>x</sub> 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 (Espenmache 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<sub>2</sub>-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 gspx 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 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 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.

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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 the presence 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.

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 (Antony 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 (MATa sec16–2 ura2–52 his4–619) was used for the preparation of ER-enriched microsomes (Wuestehube and Schekman, 1992), and FY3 strain (MATa/Mata ade2–1/ade2 can1–100/CAN1 his3–1,15/his3–2,200 leu2–3/leu2–3,112 lys2–801/LYS2 prhA16k/PRB1 pep4–6/TRP4/pep4–6HIS5 trpl–1/trpl–1 ura3–1/ura3–2) 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. Ribbons red DPH and Sypro red were purchased from Molecular Probes. 32P-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 Sec23p 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 NH2-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 the plasmid 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 (CGAGCTCAGTCGACGAGCCATCTTTG) and FS55 (GGCGCGAGCTCAGTCGACGAGCCATCTTTG) primers and was confirmed using antibodies directed against MBP (New England Biolabs, Inc.) and Sec16p.

#### Purification of Sec16p

A culture of FY3 grown in YPD broth was used to inoculate SC medium supplemented with 0.1% (v/vol) Casamino acids (Difco) and 4 mM CuSO4. Cells were grown at 30°C in a 2011 New Brunswick fermenter and harvested at an OD600 of 1.5. Cells were centrifuged, washed twice with distilled water, once with buffer JR (20 mM Heps-KOH, pH 7.4, 50 mM KOAc, 2 mM EDTA, 0.2 M sorbitol), 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 N2 and stored at ~80°C. The frozen cell suspension was mixed with 1 to 5 m/l of a cell lysate Waring blender to prepare a cell suspension. 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/m, 1 mM PMSE, and 0.1 mM TPCK). Unbroken cells and large membrane ag-
agarates 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 BB (20 mM Hepes-KOH, pH 6.8, 150 mM KOAc, 5 mM MgCl₂ [OAc], 1 mM EDTA, 4 mM DTT) for 10 min at 4°C. The stripped membranes were resuspended in 200 ml of BB8, frozen in liquid N₂, 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 BB8 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 amyllose (New England Biolabs, Inc.) column equilibrated with BB8 supplemented with PIC and 0.3 M NaCl, and the unbound proteins were removed by washing the column with 100 ml of BB8 containing PIC and 0.3 M NaCl. Therefore, the column was washed with 50 ml of BB8 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 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 N₂, 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 15,000 rpm, for 5 min at 4°C (Tomy Tech USA Inc.), washed twice with 250 ml of B88 and resuspended in 90 ml of each fraction was collected and processed for thin-section electron microscopy as described previously (Matsuoka et al., 1998).

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