Nucleation of COPII Vesicular Coat Complex by Endoplasmic Reticulum to Golgi Vesicle SNAREs

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Protein trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus involves specific uptake into coat protein complex II (COPII)–coated vesicles of secretory and of vesicle targeting (v-SNARE) proteins. Here, two ER to Golgi v-SNAREs, Bet1p and Bos1p, were shown to interact specifically with Sar1p, Sec23p, and Sec24p, components of the COPII coat, in a guanine nucleotide–dependent fashion. Other v-SNAREs, Sec22p and Ykt6p, might interact more weakly with the COPII coat or interact indirectly by binding to Bet1p or Bos1p. The data suggest that transmembrane proteins can be taken up into COPII vesicles by direct interactions with the coat proteins and may play a structural role in the assembly of the COPII coat complex.

Secretory proteins travel from the ER to the Golgi apparatus in transport vesicles coated with the COPII protein complex (*1*). The *Saccharomyces cerevisiae* COPII proteins Sar1p [a small guanosine triphosphatase (GTPase)], Sec23–24p, and Sec13–31p are necessary and sufficient to drive COPII vesicle formation in vitro from isolated ER membranes (*2*) and from liposomes composed of pure lipids (*3*). COPII vesicles also contain integral type II membrane proteins termed v-SNAREs that aid in their targeting (*4, 5*). Secretory proteins and v-SNAREs (together termed cargo proteins) are specifically sorted into COPII vesicles and enriched there to concentrations greater than in the ER (*5, 6*). When COPII budding reactions are carried out in vitro, addition of Sar1p and Sec23–24p to microsomal membranes leads to formation of complexes that contain Sar1, Sec23, and Sec24 proteins, secretory proteins and v-SNAREs, but not ER-resident proteins (*7, 8*). Thus, the cargo-sorting process takes place early in COPII vesicle formation, and the processes of COPII binding to the ER membrane and cargo recruitment may be linked.

To examine whether recruitment of the ER to Golgi v-SNAREs Bet1p, Bos1p, Sec22p, and Ykt6p (*9*) into COPII vesicles may be attributable to their direct binding to components of the COPII coat, we expressed their cytosolic domains in *Escherichia coli*, fused to glutathione S-transferase (GST) (*10*). Interaction of these fusion proteins with purified Sar1p and Sec23–24p was then investigated: The proteins were incubated together in the presence of the nonhydrolyzable GTP analog guanylyl-imidodiphosphate (GMP-PNP); the complexes formed were recovered on glutathione agarose (GSHA) beads and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (*11*). We observed strong binding of Sar1p and Sec23–24p to Bet1p and Bos1p GST fusions but not to Ykt6p or Sec22p fusions or to GST alone. These results did not depend on the location of the GST domain in the Bet1p and Sec22p fusion proteins (Fig. 1). GST–Sar1p by itself was unable to recruit Sec23–24p to GSHA beads even though it bound additional Sar1p from the solution. Thus, Sec23–24p binding to the v-SNARE was not simply a consequence of association via Sar1p.

Direct binding of vesicular coat proteins to transmembrane cargo proteins has been demonstrated for COPI vesicles (*12*) and for the AP-1 and AP-2 proteins of clathrin-coated vesicles (*13*). Although the GTP-binding protein, ADP–ribosylation factor (ARF), has been implicated in the assembly of clathrin and COPI-coated vesicles (*14*), no direct role for ARF in cargo or SNARE recruitment has been reported. This laboratory has previously proposed a model for the involvement of Sar1p and Sec23–24p in COPII cargo recognition (*7, 15*). The GDP-bound form of Sar1p is generally assumed to be recruited to the ER membrane through association with the guanine nucleotide exchange factor Sec12p, after which Sar1p·GTP mediates binding of Sec23–24p to the membrane (*3, 16, 17*). We investigated the nucleotide requirements for Sar1p and Sec23–24p binding to Bet1p fusions (Fig. 2A). Efficient binding of Sec23–24p depended on the presence of Sar1p and the GTP analog GMP-PNP. In contrast, binding of Sar1p to Bet1p was independent of Sec23–24p or the type of guanine nucleotide used, demonstrating that Sar1p binding precedes binding of Sec23–24p. The same results were obtained with GST–Bos1p.

If Sar1p·GDP can already bind to the membrane proteins Bet1p and Bos1p, how does it interact with Sec12p for nucleotide exchange? Suppose that soluble Sar1p·GDP is shielded from interaction with the v-SNAREs by a (hypothetical) Sar1p-specific GDP-dissociation inhibitor, and that Sec12p facilitates release from this interaction to allow subsequent binding to Bet1p or Bos1p. In this case, Sec12p would remain the only point of entry for Sar1p onto

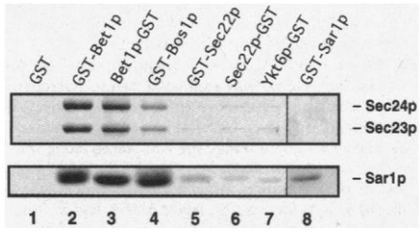

Fig. 1. Sar1p and Sec23–24p bind to the cytosolic domains of Bet1p and Bos1p. SDS-PAGE analysis of in vitro binding of Sec23–24p (upper panel) and Sar1p (lower panel) to GST (lane 1), GST fusions of v-SNAREs (lanes 2 through 7), and GST–Sar1p (lane 8). All reactions contain Sar1p and GMP-PNP. In GST–Bet1p and GST–Sec22p, GST is the NH_2-terminal fusion partner, whereas in Bet1p–GST and Sec22p–GST it is in the COOH-terminal position.

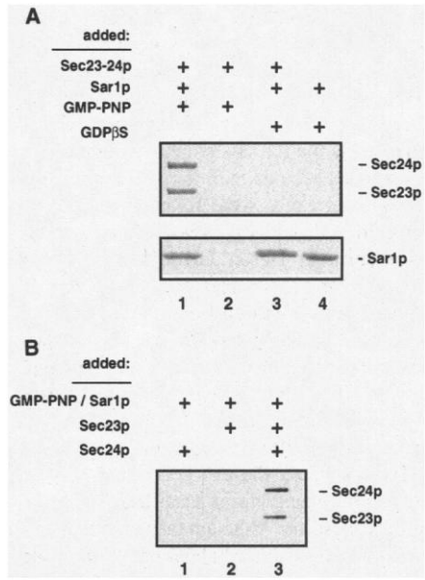

Fig. 2. Binding of Sec23 and Sec24 proteins to the cytosolic domain of Bet1p requires both subunits of the Sec23–24 complex, Sar1p, and GMP-PNP, whereas Sar1p binding to Bet1p is independent of Sec23–24p and nucleotide. (**A**) Binding of Sec23–24p (upper panel) and Sar1p (lower panel) to GST–Bet1p in reactions containing both Sar1p and GMP-PNP and lacking Sar1p, GMP-PNP, or Sec23–24p. (**B**) Binding of Sec23p (lane 1), Sec24p (lane 2), and a mixture of both individual proteins (lane 3) to Bet1p–GST.

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the ER membrane. Alternatively, Sec12p may facilitate guanine nucleotide exchange on Sar1p in a Sar1p·GDP–v-SNARE complex. In this case, the v-SNAREs would serve as additional recruiting factors for Sar1p·GDP onto the ER membrane.

GTP supported Sec23–24p recruitment onto the Sar1p–Bet1p complex much less efficiently than GMP-PNP did (*18*), probably because of the intrinsic GTPase-activating function of Sec23p for Sar1p. When Sec23p but not Sec12p is present in an in vitro reaction, guanine nucleotide exchange is the limiting step in the Sar1p–GTPase cycle (*17*). Thus, in a binding experiment containing GTP, most of the Sar1p molecules are expected to be in the GDP state and unavailable for Sec23–24p binding.

Both Sec23 and Sec24 proteins were necessary for the binding reaction. Added alone, neither protein bound detectably to the v-SNARE GST fusions in the presence of Sar1p (Fig. 2B). From titration experiments we estimated the dissociation constant for Sec23–24p from the Bet1p–Sar1p complex to be $K_d \approx 100$ nM; in the absence of Sar1p or GMP-PNP, the affinity was too low to be quantified in our system ($K_d > 1$ μM). On sucrose gradients, the peak of Sec23–24p sedimentation is shifted by about 50 to 100 kDa in the presence of Bet1p–GST, Sar1p, and GMP-PNP (corresponding to ~300 kD), which suggests a stoichiometry of one Sec23–24p heterodimer per Bet1p–GST in the complex (*18*).

Binding of Sec13–31p, the remaining element of the COPII coat, to Bet1p depended on the presence of both Sar1p and Sec23–24p (Fig. 3). The recruitment of COPII proteins to Bet1p thus takes place in the same sequence as onto microsomal membranes or liposomes (*3*). In vivo, therefore, interaction between v-SNAREs and COPII coat proteins may direct the nucleation of COPII budding sites where v-SNAREs (and possibly other cargo destined for COPII vesicles) are present, and may help anchor the emerging coats to the membrane.

In the cytosolic tails of some type I transmembrane proteins, two different motifs are required (but not sufficient) for exit from the ER in vivo, and for binding of Sec23p from cytosol in vitro: a double phenylalanine (Phe-Phe) motif, present in most members of the p24 family and in ERGIC-53, and a diacidic (Asp/Glu)-X-(Glu/Asp) motif, found in the vesicular stomatitis virus G protein (*19*). None of these sequences is present in the cytosolic part of the type II membrane protein Bet1p. We investigated which truncations of Bet1p–GST were still able to bind Sec23–24p and Sar1p (Fig. 4). Both Sar1p and Sec23–24p bound to the 79 COOH-terminal amino acids fused to GST [termed Bet1(41–119)p–GST], although Sec23–24p binding to this fragment was noticeably weaker than to the full length of the cytosolic domain. Binding to Bet1(1–65)p–GST was not detectable. Further dissection of the 41 to 119 binding region showed a lack of binding of Sec23–24p to either Bet1(41–79)p–GST or Bet1(79–119)p–GST or to a mixture of these two fusion proteins. In contrast, Sar1p bound to the COOH-terminal 41 amino acids [Bet1(79–119)p–GST], but not to Bet1(41–79)p–GST. Thus, Bet1p needs two distinct sites to interact with Sec23–24p. One of these (in residues 79 to 119) binds directly to Sar1p. The second site (in residues 41 to 79) either binds directly to Sec23–24p to form a cooperative ternary complex, or possibly elicits a conformational change in Sar1p, enabling it to bind Sec23–24p. The second site function is fulfilled more efficiently by residues 1 to 79, suggesting that the truncation boundary between residues 40 and 41 lies within the second site, or that another Sec23–24p binding site lies in residues 1 to 40. The concept that a site additional to Sar1p is necessary for Sec23–24p binding is corroborated by the findings that Sar1p immobilized to GSHA beads cannot bind Sec23–24p (Fig. 1), and that liposomes composed of the neutral lipids phosphatidylcholine and phosphatidylethanolamine bound Sar1p but subsequently failed to recruit Sec23–24p (*3*) unless negatively charged lipids were present. The necessity for at least two spatially distinct sites on a protein to recruit Sec23–24p would help explain why a transplantable ER export motif for type II transmembrane proteins has been difficult to identify.

Proteins that do not bind Sar1p (for example Sec22p and Ykt6p) cannot recruit Sec23–24p (Fig. 1). How are those membrane proteins recruited into COPII vesicles? Either they may exhibit weaker interactions with COPII proteins, which our assay is unable to detect, or they may bind to proteins that can interact with the COPII coat. For Sec22p, this is probably a protein that is not removed by washing membranes with urea (*7*). Bet1p and Bos1p are candidates for such an intermediary. Interaction of Sec22p with Bos1p has been demonstrated with recombinant proteins

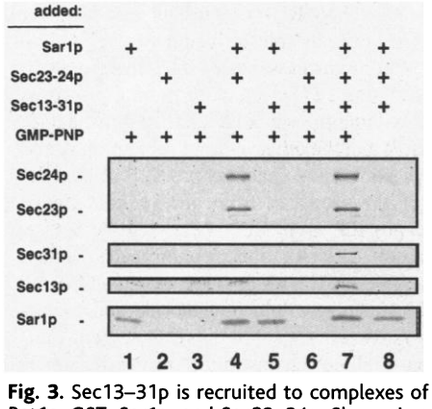

Fig. 3. Sec13–31p is recruited to complexes of Bet1p–GST, Sar1p, and Sec23–24p. Shown is a binding experiment with different combinations of COPII components to Bet1p–GST in the presence of GMP-PNP (lanes 1 through 7) or GDPβS (lane 8). In this experiment, 0.01% Triton X-100 was used instead of octyl glucoside to prevent nonspecific binding of Sec13–31p.

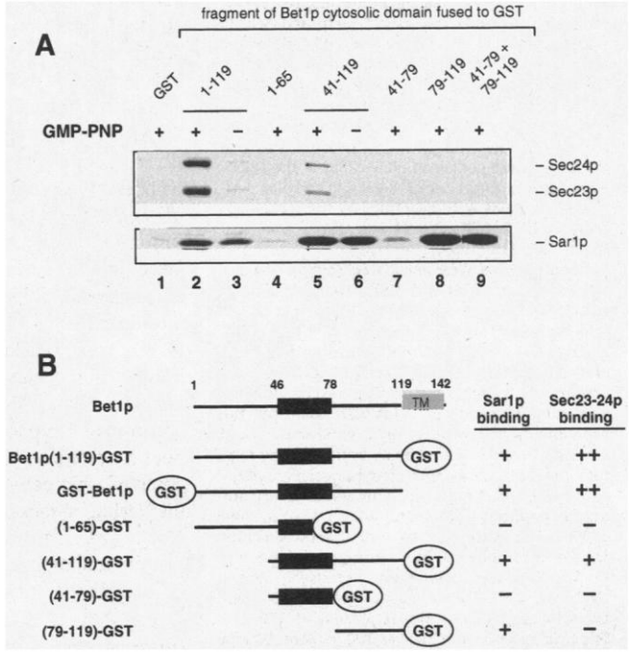

Fig. 4. (**A**) Sec23–24p binding is localized to the COOH-terminal (membrane-proximal) 79 amino acids of the cytosolic domain of Bet1p, whereas Sar1p can bind to the 41 COOH-terminal amino acids of the cytosolic domain. Shown is the binding of Sec23–24p to Bet1(1–119)p–GST (the full length of the cytoplasmic domain fused to GST), Bet1(1–68)p–GST, Bet1(41–119)p–GST, Bet1 (41–79)p–GST, Bet1(79–119)p–GST, and a mixture of Bet1(41–79)p–GST and Bet1(79–119)p–GST, in the presence or absence of GMP-PNP. Samples without GMP-PNP contained an equivalent amount of GDP-β-S. Sar1p was added to all samples. (**B**) Schematic depiction of Bet1p and GST fusions of Bet1p and summary of binding data from Fig. 1 and (A). TM, transmembrane domain. The filled-in boxes correspond to the predicted helical (and potential coiled-coil) domains (*22*).

(*20*). We have also observed specific interactions between a recombinant histidine-tagged Bet1p cytosolic domain and both GST–Sec22p and Ykt6p–GST, but not GST alone (*18*). Alternatively, some other membrane protein or lipid may assist in recruiting GST–Sec22p and Ykt6p.

Cargo proteins and v-SNAREs are concentrated in designated regions of the ER during the COPII budding process (*5–7, 21*). Our observations suggest that v-SNAREs help to direct COPII vesicle nucleation to such regions and are thus themselves included in the emerging COPII vesicles. Other transmembrane cargo proteins, including receptors for ER-lumenal cargo, may initially be present in an association with the v-SNAREs or may become included through their own interactions with the COPII coat.

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10. Fusions of the cytosolic domains of the v-SNAREs to the COOH- or the NH_2-terminus of GST were expressed in *E. coli* from the vectors pGEX-2T (Pharmacia) and pETGEXCT [A. D. Sharrocks, *Gene* **138**, 105 (1994)] and purified as described [J. V. Frangioni and B. G. Neel, *Anal. Biochem.* **210**, 179 (1993)]. They were exchanged into binding buffer (BB; see below) containing glycerol, 150 μl/ml. Sar1p and Sec23p were expressed as GST fusions in *E. coli* and isolated and cleaved from the GST domain as described [(7); E. Gimeno, P. Espenshade, C. A. Kaiser, *Mol. Biol. Cell* **7**, 1815 (1996)]. Sec23–24(His_6)p and Sec13–31(His_6)p complexes were isolated from *S. cerevisiae* as described (*7*). Sec24(His_6)p was obtained from wash fractions (which contained no Sec23p) of the anion-exchange step in the Sec23–24(His_6)p complex purification. Proteins were quantified by tryptophan absorbance [S. C. Gill and P. H. von Hippel, *Anal. Biochem.* **182**, 319 (1989)].
11. Purified proteins were incubated together at the following concentrations: GST fusion proteins, 3 μM; Sar1p, 1.6 μM; Sec23–24p, 170 nM; in BB [20 mM Na Hepes, pH 6.8, 5 mM $MgCl_2$, 1 mM EDTA, glycerol (20 μl/ml), 300 mM potassium acetate, 1 mM dithiothreitol (DTT)] containing 10% lipid emulsion or 0.2% octyl glucoside. Results obtained with octyl glucoside or lipids were identical; the experiments shown use lipid emulsions. [Lipid emulsions were prepared by hydrating a dried mixture of 2 mg of dipalmitoleoyl-phosphatidylethanolamine and 3 mg of dipalmitoleoyl-phosphatidylcholine (both from Avanti Polar Lipids) in 1 ml of BB and sonicating on ice with a probe sonicator for 4 × 30 s, followed by centrifugation for 5 min at 15,000*g*; the supernatant was used.] Where indicated, GMP-PNP or guanosine 5′-*O*-(2′-thiotriphosphate) (GDP-β-S) was present at 170 μM. The total reaction volume was 100 μl. After 90 min of rotation at 4°C, the reaction mixture was added to 15 μl of prewashed GSHA beads, and the incubation was continued for 30 min more. Beads were washed 3× with BB and 1× with 20 mM Na Hepes, pH 6.8, and then heated to 65°C for 10 min in SDS sample buffer. Eluted proteins were analyzed by SDS gel electrophoresis and Coomassie Blue R-250 staining.
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Functional Reconstitution of Ypt7p GTPase and a Purified Vacuole SNARE Complex

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Membrane trafficking has heretofore been studied with intact organelles. Here, fusion-competent proteoliposomes were reconstituted from a yeast vacuole detergent extract. Homotypic vacuole fusion requires many membrane proteins, including the Ypt7p guanosine triphosphatase and a "SNARE complex" with Vam3p and Nyv1p. Proteoliposomes from extracts immunodepleted of either Vam3p or Ypt7p could not fuse, but vesicles reconstituted from a mixture of these depleted extracts had restored fusion activity. Purified preassembled vacuolar SNARE complex, when reconstituted with a SNARE-depleted extract, was fully functional for fusion. Thus, solubilized integral membrane components can be reconstituted for priming, docking, and fusion steps of organelle trafficking.

Fusion of opposing membranes (*1*) requires interactions between integral membrane SNARE proteins. *N*-Ethylmaleimide–sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs) bind to the SNARE complex and catalyze its disassembly (*2, 3*). Rab/Ypt guanosine triphosphatases (GTPases) may modulate SNARE associations (*3, 4*). An in vitro assay of homotypic vacuole fusion (*5, 6*), the final step of vacuole inheritance in *Saccharomyces cerevisiae*, requires Sec18p (yeast NSF), Sec17p (alpha-SNAP) (*7*), a complex of the SNAREs Vam3p and Nyv1p (*8, 9*), Ypt7p (GTPase) (*10*), and LMA1 (*11*). The reaction occurs in steps of priming, docking, and fusion. Priming entails Sec18p and adenosine triphosphate (ATP)–mediated Sec17p release (*12*), dissociation of the SNARE complex, and activation of Vam3p. Docking, the stable association of vacuoles, requires Ypt7p (*10, 13*). Fusion itself requires an unidentified phosphatase.

Membrane trafficking requires SNAREs on separate vesicle (v-SNARE) and target organelle (t-SNARE) membranes to bind (*1*). Nonetheless, "cis" complexes of v- and t-SNAREs are found on secretory vesicles, endoplasmic reticulum to Golgi vesicles (*14*), and vacuoles (*9*). Purification of a functional vacuolar SNARE complex is essential for determining its role in fusion.

Our in vitro assay used vacuoles from two yeast strains (*15*), one with normal proteases but without the vacuolar alkaline phosphatase (ALP) and the other without proteinase A and thus containing inactive proALP. Upon fusion, the proteases from one vacuole cleave the proALP from the other, yielding active, mature ALP for spectrophotometric assay. Vacuoles (*15*) with proALP were solubilized with *n*-octyl-β-D-glucopyranoside and asolectin (soybean phospholipids). Detergent dilution yielded reconstituted proteoliposomes (*16, 17*) with a protein composition similar to that of vacuoles (Fig. 1A) and having membrane proteins Sec18p, Ypt7p, and Sec17p and the SNAREs Vam3p and Nyv1p but little lumenal CPY (carboxypeptidase Y; Fig. 1B). Half of the Sec17p on reconstituted proteoliposomes was accessible to added protease

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