(*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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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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23. We thank C. Kaiser for the bacterial GST–Sec23p expression clone, S. Ferro-Novick for the His_6-Bet1p and His_6-Bos1p expression clones, R. Lesch for the preparation of some proteins, and members of the Schekman laboratory for improvements to the manuscript.

29 April 1998; accepted 22 June 1998

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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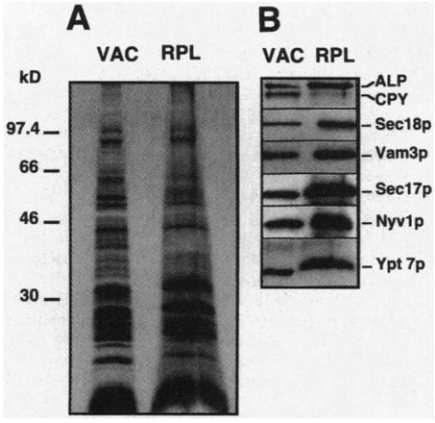

Fig. 1. Reconstitution of proteoliposomes from detergent-solubilized vacuoles. (**A**) BJ3505 vacuoles (20 μg; VAC) or reconstituted proteoliposomes (RPL) were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and silver staining. (**B**) VAC and RPL were analyzed by immunoblotting with antibodies to ALP, CPY, Sec18p, Vam3p, Sec17p, Nyv1p, and Ypt7p.

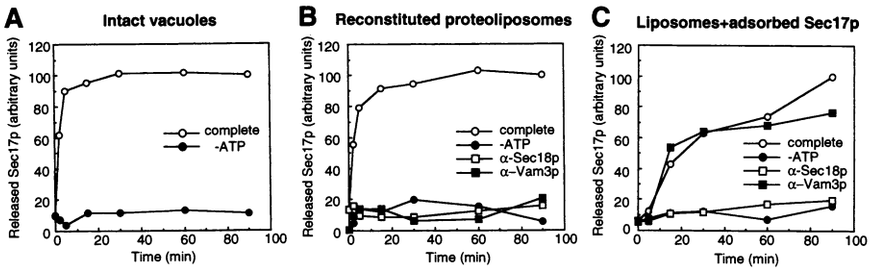

Fig. 2. Reconstituted vesicles support priming. Released Sec17p (*12*) from 5×-volume standard assays. (**A**) Thirty micrograms of vacuoles; (**B**) 15 μg of reconstituted vesicles. Portions were diluted 10-fold with buffer [10 mM Pipes-KOH (pH 6.8) containing 200 mM sorbitol, 150 mM KCl, 5 mM EDTA, and 1 mM PMSF], the membranes were removed by centrifugation (10,000*g*, for 5 min or 125,000*g* for 30 min), and the released Sec17p was assayed by precipitation with trichloroacetic acid (TCA), SDS-PAGE, immunoblotting, and quantification. (**C**) Purified His_6-Sec18p (1 μg) and His_6-Sec17p (0.8 μg) were incubated with protein-free asolectin liposomes [1.25 mg, prepared (*16*) without vacuoles or proALP] in fusion reaction buffer (150 μl) without ATP on ice for 15 min. Liposomes were collected, resuspended in this buffer with ATP (*12*), and assayed for released Sec17p as above, with an ether wash to remove excess lipids. For antibody concentrations, see legend to Fig. 3. α-Sec18p and α-Vam3p, antibodies to Sec18p and Vam3p.

(*18*), suggesting that only half of this and other reconstituted components is physiologically oriented and hence functional.

Sec17p undergoes ATP-dependent release from vacuoles (Fig. 2A) during priming (*12*). Reconstituted proteoliposomes also supported ATP-dependent release of the endogenous Sec17p (Fig. 2B), but this was blocked by antibody to Sec18p or Vam3p. Because all of the Sec17p in this experiment came from solubilized vacuole membranes, the inhibition of Sec17p release by antibody to Vam3p indicates that Sec18p catalysis occurred at SNARE sites (*9*) that were preserved during solubilization and reconstitution. In contrast, the Sec18p-dependent release of Sec17p that had been adsorbed to protein-free asolectin liposomes was not inhibited by antibody to Vam3p (Fig. 2C), again indicating that the priming of reconstituted proteoliposomes in the experiment of Fig. 2B had occurred at functional SNARE sites. This loss of Sec17p affinity for lipid (Fig. 2C) also suggests that Sec18p action alters the conformation of Sec17p.

Encouraged by this reconstitution of priming, we asked whether these proteoliposomes could fuse with intact vacuoles. Reconstitution was performed with His_6-proALP, purified from *Escherichia coli* (*19*), and the vacuole detergent extract, which contained endogenous proALP. Proteoliposome fusion activity, measured as proALP maturation by lumenal protease A (*20*) from intact vacuoles, increased with added His_6-proALP (Fig. 3A), indicating that the added and endogenous proenzyme had mixed in the detergent extract. The authenticity of this reconstituted fusion was shown by its dependence on ATP and physiological temperature and by its sensitivity to Gdi1p (which extracts Ypt7p) or to antibodies to Sec17p, Sec18p, Ypt7p, or Vam3p (Fig. 3B) (*8, 12*). Up to 2.0% of the reconstituted proALP was matured after the fusion reaction. Because only 50% of the His_6-proALP was correctly oriented and because fusion reactions with intact vacuoles processed ~40% of the proALP to ALP, the yield of fusion activity during vacuole solubilization and reconstitution was 10%. The multiplicative effect of loss of transmembrane asymmetry of the SNARE complex, Ypt7p, the phosphatase, and other factors during reconstitution may well account for this yield. However, these data do not establish whether each protein that supports trafficking was required on both the proteoliposomes and the intact, partner vacuoles or whether some inhibitors (Fig. 3B) affected only the intact vacuoles.

To test this, we prepared a vacuole detergent extract that was immunodepleted with immobilized preimmune immunoglobulin G (IgG) or IgG to Vam3p or Ypt7p. Over 90% of Vam3p or Ypt7p was removed by this procedure, with little loss of the complementary protein (Fig. 4A). Sec17p and Nyv1p are also present in the SNARE complex (*9*), and ~70% of the Sec17p and 40% of Nyv1p were co-depleted by antibody to Vam3p (*18*). When either depleted extract was reconstituted into proteoliposomes, fusion activity was lost (Fig. 4B). However, fusion activity was recovered by co-reconstitution of a mixture of both detergent extracts (Fig. 4B). Because the amounts of Ypt7p or Vam3p were not affected by immunodepleting Vam3p or Ypt7p, respectively (Fig. 4A), these data suggest that Ypt7p is not a stable component of the yeast vacuole SNARE complex, that both components are required in proteoliposomes for fusion, and that functional SNARE complex and G protein may be purified separately.

As a first step toward establishing a reconstituted system with pure components, we isolated the SNARE complex (*21*). Although separately purified recombinant SNAREs support a slow fusion re-

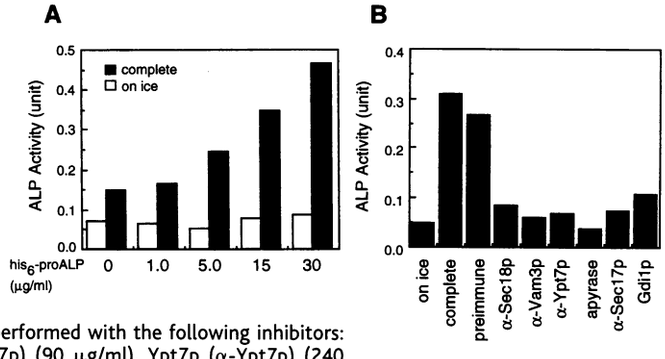

Fig. 3. Authenticity of fusion. (**A**) Reconstituted proteoliposomes (10 μl, 5 μg, with His_6-proALP) were fused (*6*) with intact DKY6281 (ΔPHO8) vacuoles for 90 min at 27°C with α_2-macroglobulin (10 μg/ml) and cytosol (1 mg/ml). The reaction was centrifuged (16,000*g* for 10 min), and the pellets were assayed for ALP (*6*). (**B**) Fusion reactions were performed with the following inhibitors: IgG to Sec17p (α-Sec17p) (90 μg/ml), Ypt7p (α-Ypt7p) (240 μg/ml), or Vam3p (α-Vam3p) (200 μg/ml); affinity-purified antibody to Sec18p (α-Sec18p) (80 μg/ml); Gdi1p (180 μg/ml); and apyrase (10 U/ml). Fusion-independent proteoliposomal phosphatase activity was subtracted in (A) (0.060 to 0.137 unit) and (B) (0.063 unit).

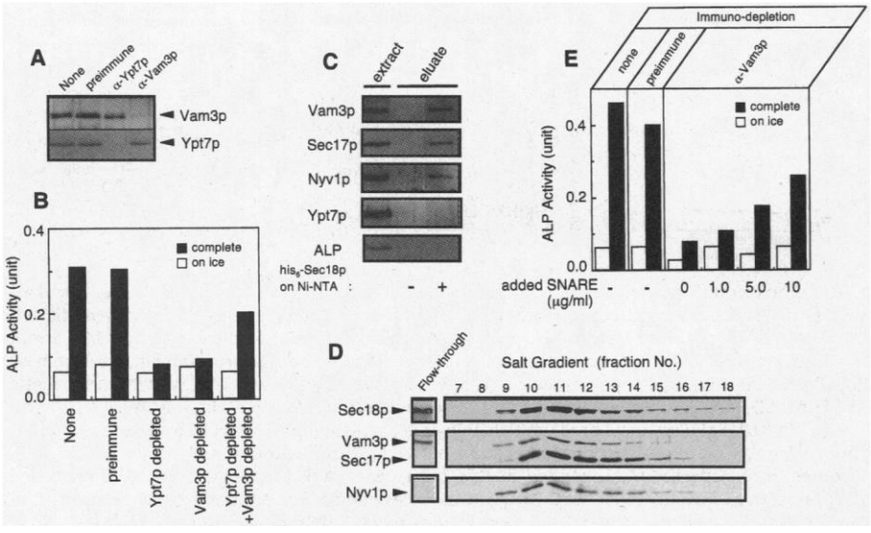

Fig. 4. Both the SNARE complex and Ypt7p are required for fusion. **(A)** Vacuole detergent extract was either untreated (none) or immunodepleted (180 min at 4°C) with protein A–Sepharose bearing preimmune IgG or antibodies to Vam3p (α-Vam3p) or Ypt7p (α-Ypt7p). Each portion was TCA-precipitated and analyzed by SDS-PAGE and immunoblotting. **(B)** Reconstituted proteoliposomes from (A) were analyzed for fusion (180 min) with DKY6281 vacuoles as in Fig. 3. "Ypt7p depleted" or "Vam3p depleted" indicates proteoliposomes from extracts depleted with antibodies to Ypt7p or Vam3p, respectively. "Ypt7p depleted + Vam3p depleted" indicates vesicles reconstituted from a mixture of equal amounts of each immunodepleted detergent extract. Fusion-independent phosphatase activity of 0.068 to 0.083 unit was subtracted. **(C)** SNARE complex purification on His_6-Sec18p Ni-NTA or Ni-NTA agarose alone was as described (*21*). Detergent extract and eluents were precipitated with TCA and analyzed by SDS-PAGE and immunoblotting. **(D)** SNARE complex purification. The His_6-Sec18p imidazole eluate was bound to a 5-ml DEAE-Sephacel column in buffer B, washed, and eluted (40 fractions, 50 to 500 mM NaCl gradient in 40 ml of buffer B). Shown are the flow-through and peak fractions after SDS-PAGE and immunoblotting. **(E)** Proteoliposomes from mixtures of purified SNARE complex (as above, but 0.2 M NaCl step elution from DEAE) and a Vam3p-immunodepleted detergent extract were assayed (180 min) for fusion with vacuoles from DKY6281 as in Fig. 3. "None" or "preimmune" indicates proteoliposomes from extracts that were not immunodepleted or that were mock-depleted with preimmune antibody, respectively. Fusion-independent phosphatase activity of 0.060 to 0.085 unit was subtracted, as in Fig. 3. α-Vam3p, antibody to Vam3p.

action (*22*), a purified complex of v- and t-SNAREs has not been shown to catalyze Rab- or Ypt-dependent fusion. Vacuolar detergent extract was preadsorbed with nickel–nitrilotriacetic acid (Ni-NTA) modified agarose and was then incubated with Ni-NTA agarose bearing purified His_6-Sec18p (Fig. 4C). The beads were washed, and the bound material was eluted with imidazole. The eluent contained the SNARE complex components Vam3p, Nyv1p, Sec18p, and Sec17p but not Ypt7p or proALP. The SNARE complex was freed from unbound Sec18p and uncomplexed Vam3p by ion-exchange chromatography (Fig. 4D). This purified complex needs only ATP and LMA1 for "priming" dissociation (*18*). Addition of this SNARE complex, ~150-fold purified from vacuoles, to the SNARE-depleted detergent extract restored the fusion activity of the resulting proteoliposomes (Fig. 4E). These proteoliposomes had 83% of the fusion activity and 94% of the Vam3p content of proteoliposomes from the control extract.

These results demonstrate solubilization and reconstitution of the membrane fusion step of trafficking. This has allowed a direct biochemical demonstration that the cis complexes of v- and t-SNAREs on the same (vacuole) membrane (*9*) are fully functional for Sec18p and ATP-dependent dissociation and activation during priming and for docking and fusion, suggesting that the v- and t-SNARE complexes found on synaptic vesicles (*23*) might also be functional.

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16. Solubilization and reconstitution: Vacuoles [0.3 mg/ml, freshly isolated (*6*)] were solubilized for 20 min at 0°C in 20 mM Hepes-KOH (pH 7.4), containing 150 mM NaCl, 2 mM dithiothreitol (DTT), 10% (v/v) glycerol, protease inhibitor cocktail (PIC) (*11*), 1.25% (w/v) *n*-octyl-β-D-glucopyranoside, asolectin (12.5 mg/ml) and His_6-ALP. The clarified (20 min, 125,000*g*) extract was rapidly diluted with 40 volumes of 20 mM Hepes-KOH (pH 7.4) containing 150 mM NaCl and incubated (4°C, 10 to 15 min, gentle mixing). Reconstituted proteoliposomes were sedimented (55,000 rpm, for 60 min at 4°C, with a Beckman 60Ti rotor), suspended to 0.5 volumes in 10 mM Pipes-KOH (pH 6.8) containing 200 mM sorbitol, frozen in liquid nitrogen, thawed, sonicated (5 to 10 s in a Branson bath sonifier, model 2200), and centrifuged (16,000*g*, 10 min). The supernatant was reconstituted proteoliposomes.
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25. We thank N. Margolis for expert assistance and R. Schekman, C. Barlowe, Z. Xu, C. Ungermann, G. Eitzen, J. Eichler, F. Duong, and A. Price for discussions. Supported by the National Institute of General Medical Sciences. K.S. is supported by a Japan Society for the Promotion of Science Fellowship and an Human Frontier Science Program Long-Term Fellowship.

17 April 1998; accepted 22 June 1998