Sec12p-dependent Membrane Binding of the Small GTP-binding Protein Sar1p Promotes Formation of Transport Vesicles from the ER

Christophe d'Enfert, Linda J. Wuestehube, Tom Lila, and Randy Schekman

Division of Biochemistry and Molecular Biology, and Howard Hughes Medical Institute, Barker Hall, University of California, Berkeley, California 94720

Abstract. Sec12p is an integral membrane protein required in vivo and in vitro for the formation of transport vesicles generated from the ER. Vesicle budding and protein transport from ER membranes containing normal levels of Sec12p is inhibited in vitro by addition of microsomes isolated from a Sec12p-overproducing strain. Inhibition is attributable to titration of a limiting cytosolic protein. This limitation is overcome by addition of a highly enriched fraction of soluble Sar1p, a small GTP-binding protein, shown previously to be essential for protein transport from the ER and whose gene has been shown to interact genetically with *sec12*. Furthermore, Sar1p binding to isolated membranes is enhanced at elevated levels of Sec12p. Sar1p–Sec12p interaction may regulate the initiation of vesicle budding from the ER.

GENETIC analysis has identified four yeast genes that are required for the formation of transport vesicles derived from the ER: *SEC12*, *SEC13*, *SEC16*, and *SEC23* (Kaiser and Schekman, 1990). In vitro reconstitution of ER-Golgi transport vesicle formation also showed a requirement for Sec12p and Sec23p (Rexach and Schekman, 1991). Cell fractionation and DNA sequence analysis have shown that Sec12p is an integral membrane protein, and Sec23p, Sec16p, and Sec13p are either cytosolic or peripheral membrane proteins (Nakano et al., 1988; Hicke and Schekman, 1989; Kaiser, C., and N. Pryer, unpublished data). Interactions observed among the genes encoding these proteins suggest that they could act in concert or form a multisubunit complex possibly associating with the ER membrane via Sec12p (Kaiser and Schekman, 1990).

Sec12p is a type II membrane glycoprotein with a 40 kD NH_2-terminal cytosolic domain that is essential for transport, and a COOH-terminal lumenal domain that is dispensable (Nakano et al., 1988; d'Enfert, C., C. Barlowe, S. Nishikawa, A. Nakano, and R. Schekman, manuscript submitted for publication). The thermosensitive lethality associated with a mutation in the NH_2-terminal domain of Sec12p is suppressed by overproduction of Sar1p, a 21-kD GTP-binding protein (Nakano and Muramatsu, 1989). Sar1p is itself required for protein transport from the ER (Nakano and Muramatsu, 1989). Cell fractionation experiments show Sar1p both in the cytosol and in tight association with a membrane fraction, however overproduction increases the fraction of Sar1p soluble in the cytosol (Nishikawa and Nakano, 1991). Membrane association of Sar1p is enhanced in vivo by overproduction of Sec12p (d'Enfert, C., C. Barlowe, S. Nishikawa, A. Nakano, and R. Schekman, manuscript submitted for publication). These results suggest a structural and functional interaction of Sar1p and Sec12p.

In the course of identifying cytosolic factors that stimulate protein transport from the ER in vitro, we observed that addition of a membrane fraction with elevated levels of Sec12p was inhibitory, and that a limiting cytosolic factor restored transport when provided in an enriched form. This report describes the identification of this limiting factor as Sar1p. Extensive purification of a functional, apparently monomeric soluble form of Sar1p is achieved.

C. d'Enfert's present address is Laboratoire de Génétique des Microorganismes, C.B.A.I., 78850 Thiverval-Grignon, France.

Materials and Methods

Strains, Plasmids, Materials, and General Methods

The yeast strains used in this study were RSY607 (*leu2-3,112 ura3-52 pep4::URA3 MATα*) and YPH500 (*ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 MATα*; Sikorski and Hieter, 1989). Plasmids pANY1-9 (2μ *URA3 SEC12*), pCEY5 (2μ *URA3 GAL1-SEC12*), pANY2-7 (2μ *URA3 SAR1*), pANY2-18 (*CEN4-ARS1 TRP1 GAL1-SAR1*), and pSEY8 have been previously described (Nakano et al., 1988; d'Enfert, C., C. Barlowe, S. Nishikawa, A. Nakano, and R. Schekman, manuscript submitted for publication; Nakano and Muramatsu, 1989; Emr et al., 1986). pCGS109 (2μ *URA3 'GAL1-GAL10'*) is a gift of D. Moir (Collaborative Research, Lexington, MA). pSEC1313 (2μ *URA3 SEC13*) and pSEC-1614 (2μ *URA3 SEC16*) were kindly provided by C. Kaiser (Division of Biochemistry and Molecular Biology, and Howard Hughes Medical Institute, University of California, Berkeley, CA).

Yeast cells were grown in YP (2% Bacto-peptone 1% Yeast extract broth both from Difco Laboratories Inc., Detroit, MI) containing 2% glucose or in MV (0.67% yeast nitrogen base without amino acid; Difco Laboratories Inc.) containing 2% glucose and supplemented with the appropriate amino acids and/or 0.5% vitamin assay Casamino acids (Difco Laboratories Inc.).

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For induction of *GAL1*-regulated genes, cells were first grown in MV containing 2% lactate, pH 5.5, 0.1% glucose, and then induced by transfer in either YP 2% galactose for 20 h or MV 2% lactate, pH 5.5, 2% galactose for 5 h.

Anti-Sec12p, anti-Sar1p, anti-Sec23p, anti-Kar2p, and anti-α,1-6-mannose antibodies have been described (Nakano et al., 1988; Nishikawa and Nakano, 1991; Hicke and Schekman, 1989; Rose et al., 1989; Baker et al., 1988). Anti-Sec13p, anti-Ypt1p, and anti-phosphoglycerokinase were kindly provided by C. Kaiser, D. Baker, and J. Thorner, respectively (Division of Biochemistry and Molecular Biology, and Howard Hughes Medical Institute, University of California, Berkeley, CA). Transfer of proteins from SDS-PAGE gels to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) was performed as described (Towbin et al., 1979). Filters were blocked and all incubations were conducted in 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% NP-40 with or without 2% non-fat dry milk. Detection of filter-bound antibodies with horseradish peroxidase-conjugated goat anti-rabbit IgG and with ECL immunoblotting reagents were performed according to the supplier's instructions (Amersham Corp., Arlington Heights, IL). Protein concentrations were determined by the procedure of Lowry et al. (1951) in the presence of 1% sodium dodecyl sulfate or with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) using BSA as a standard.

Preparation of Subcellular Fractions for In Vitro Assays

Subcellular fractions were prepared from wild-type cells grown in YP 2% glucose or from transformed cells grown in YP 2% galactose (cytosol) or MV 2% lactate, 2% galactose (cytosol, microsome, and high speed pellet fractions). Cytosol was prepared from bead-lysed cells as described (Baker et al., 1988) and concentrated (12-15 mg protein/ml) by centrifugation in Centriprep-10 unit (Amicon Corp., Danvers, MA). Preparations of microsomes and high speed pellet (HSP) fractions from osmotically-lysed cells were carried out according to Baker et al. (1990) except that glucose was replaced by galactose during the formation of spheroplasts when fractions were prepared from cells grown in the presence of galactose. Alternatively, the high speed pellet fraction was obtained after a 100,000-*g* centrifugation of a 12,000-*g* supernatant from a bead-lysed cell extract (Baker et al., 1988). This HSP fraction (20 mg protein/ml) had a lower specific transport activity but stimulated ER-Golgi transport as efficiently as an HSP fraction derived from osmotically lysed cells.

In Vitro Assays and Microsome-dependent Inhibition

Two-stage in vitro transport reactions were carried out as described (Baker et al., 1990) using ^{35}S-labeled prepro-α-factor (Baker et al., 1988), microsomes (10 μg protein), cytosol (110-150 μg protein), and HSP fractions (20-40 μg protein) in a total reaction volume of 50 μl. Transport conditions were adjusted so that activity was linearly dependent upon addition of cytosol and HSP fractions and are noted in the figure legends. Reactions were incubated for 2 h at 20°C and terminated by addition of 50 μl 2% SDS followed by heating at 95°C for 5 min. The total amount of glycosylated pro-α-factor was quantified by precipitation with concanavalin A-Sepharose; Golgi-modified forms of pro-α-factor were quantified by precipitation with an antiserum specific for (α1-6)-linked mannose and protein A-Sepharose as described (Baker et al., 1988). In these conditions, transport efficiency (ratio of Golgi-modified/total glycosylated pro-α-factor) was typically 25% and was linearly dependent upon addition of cytosol and HSP fractions.

Two-stage in vitro budding reactions were carried out using microsomes (2.5 μg protein) and cytosol (470 μg protein) in a total reaction volume of 50 μl (Wuestehube, L., and R. S. Schekman, manuscript in preparation). Reactions were incubated for 30 min at 20°C and terminated by centrifugation at 27,000 *g* for 10 min. The total reaction mixture and supernatant fraction were treated with trypsin (0.3 mg/ml, 60 min, 4°C) followed by trypsin inhibitor (1.2 mg/ml, 10 min, 4°C). Samples were heated at 95°C for 5 min in the presence of 1% SDS and the amount of glycosylated pro-α-factor was quantified by precipitation with concanavalin A-Sepharose as described (Baker et al., 1988). Efficiency of vesicle release (ratio of supernatant/total glycosylated pro-α-factor) typically was 24%.

To test the inhibitory effects of competitor membranes, microsomes (0.5-5 mg protein/ml) were treated first with trypsin (0.2 mg/ml, 30 min, 4°C) and then with trypsin inhibitor (0.5 mg/ml, 10 min, 4°C). Trypsin treatment did not affect the integrity of the ER membranes since the luminal protein Kar2p remained enclosed within the sedimentable material (not shown). Various amounts of trypsin-treated membranes were added to cytosol and HSP fractions and the concentrations of trypsin and trypsin inhibitor were adjusted accordingly. Alternatively, trypsin-treated membranes were washed by centrifugation (27,000 *g*, 10 min) in reaction buffer to remove soluble trypsin and trypsin inhibitor. After a 10-min incubation on ice, microsomes containing translocated ^{35}S-pro-α-factor were added and the second stage of the reaction was initiated. An 80% inhibition was reproducibly obtained with 4-5 μg of Sec12p-enriched membranes.

To test the rescue activity of various fractions, cytosol and HSP fractions were mixed with the appropriate amount of trypsin-treated Sec12p-enriched microsomes such that ~80% inhibition was obtained in the absence of any additional component. This mixture was kept on ice for 10 min, distributed into tubes containing the fractions to be tested, and incubated an additional 10 min on ice. Microsomes containing translocated ^{35}S-pro-α-factor then were added and the second stage of the reaction was conducted.

Gel Filtration of Yeast Cytosols

Cytosol was prepared from YPH500(pANY2-18) as described above. A 400-μl aliquot of cytosol (5.9 mg) in reaction buffer containing 1 mM DTT, 0.5 mM PMSF, 1 mM ATP, and 0.1 mM GTP was loaded onto a 20 ml (1.0 × 25 cm) Sephacryl S-100 HR (Pharmacia Inc., Piscataway, NJ) gel filtration column equilibrated in reaction buffer. The column was eluted in the same buffer at ~12 ml/h. Fractions (400 μl) were collected and total protein and rescue activity in each fraction was determined. Fractions were resolved by SDS-PAGE, visualized by silver stain (Morrisey, 1981), and examined for Sar1p content by immunoblot. As a control, cytosols not enriched in Sar1p were prepared from YPH500(pANY2-18) and YPH500-(pCGS109), adjusted to a protein concentration of 9.5 mg/ml, and fractionated using the same S-100 HR gel filtration column.

Sar1p Membrane Binding Assay

Trypsin-treated microsomes (20 μg) were prepared as described above and mixed with various fractions containing soluble Sar1p in a final reaction buffer volume of 40 μl. BSA (1 mg/ml) was included when partially purified Sar1p was used in order to avoid proteolysis. After a 5-min incubation at 4°C, the membrane fraction (Fig. 7, *MSP*) was separated from the soluble fraction (Fig. 7, *MSS*) by a 25,000-*g* centrifugation for 10 min in a TLA 100 rotor (Beckman Instruments, Inc., Palo Alto, CA). The supernatant fraction was then recovered and diluted with SDS-PAGE loading buffer (5×). The pellet was washed twice with reaction buffer and resuspended in SDS-PAGE loading buffer (1×). Both fractions were analyzed by SDS-PAGE and immunoblotting using anti-Sar1p and anti-Sec12p antibodies as well as several control antibodies raised against three peripheral membrane Sec proteins (Sec23p, Ypt1p, and Sec13p) and a cytosolic protein (phosphoglycerokinase, PGK). Displacement of soluble Sar1p into the Sar1p-depleted microsomal fraction was then assayed by comparing the Sar1p content of each fraction. Sec12p-dependent membrane binding was observed only for Sar1p and no significant membrane binding was observed with Sec23p, Ypt1p, Sec13p, and PGK.

Results

Sec12p-enriched Membranes Inhibit Transport

Protein transport from the ER to the Golgi apparatus has been reconstituted with yeast membranes and cytosol (Baker et al., 1988, 1990; Ruohola et al., 1988). In the first stage of transport, [^{35}S]methionine-labeled α-factor precursor is translocated into a crude ER membrane fraction to form core-glycosylated precursor. Transport to the Golgi complex is achieved in a second stage which measures the coupled addition of outer chain carbohydrate to the core-glycosylated precursor. In the assay developed by Baker et al. (1990), transport is stimulated by a cytosol fraction (100,000-*g* supernatant), a particulate fraction (HSP; 100,000-*g* pellet), and ATP. The particulate fraction appears to provide an enriched source of factors that are present in limited supply in the cytosol, rather than an essential membrane compartment. Addition of a salt wash soluble fraction, obtained by extraction of permeabilized spheroplasts, replaced the requirement

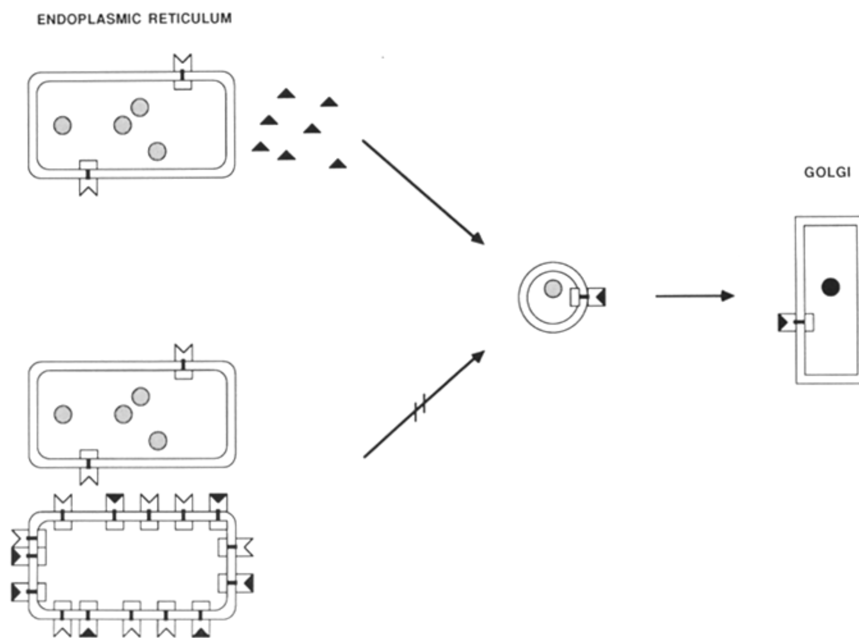

Figure 1. Inhibition of ER–Golgi transport by Sec12p-enriched membranes. In vitro-reconstituted ER–Golgi transport monitors the transfer of core-glycosylated ^{35}S-labeled pro-α-factor (*dotted circle*) from the endoplasmic reticulum to the Golgi apparatus where it acquires outer-chain glycosylation (*solid circle*). Interaction between Sec12p (membrane protein) and an unknown cytoplasmic component (*solid triangle*) is thought to be necessary for the production of a transport vesicle. Addition of Sec12p-enriched membranes to a standard transport reaction should result in a titration of this cytoplasmic component by these membranes and thus in an inhibition of transport.

for HSP material (Baker, D. and L. Wuestehube, unpublished results).

To identify cytosolic factors that interact with Sec12p, we designed an assay in which these components become limiting through titration by excess Sec12p. This was achieved by mixing wild-type ER membranes containing ^{35}S-core-glycosylated pro-α-factor with nonradioactive membranes isolated from cells transformed with a multicopy vector containing the *SEC12* gene. An outline of this approach is depicted in Fig. 1. If the inhibitory membranes consume a specific limiting component, inhibition should be overcome by supplementing the reaction with an enriched source of this limiting protein.

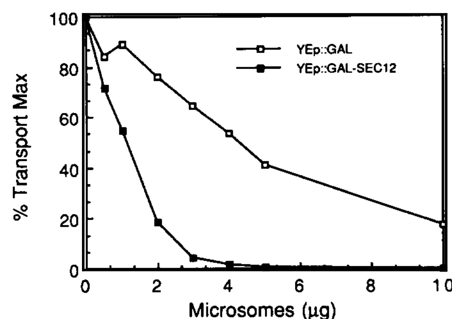

Figure 2. Sec12p-enriched membranes specifically inhibit transport. Standard ER–Golgi transport reactions were carried out with 10 μg wild-type microsomes containing translocated pro-α-factor, 110 μg wild-type cytosol, and 20 μg wild-type HSP prepared from osmotically lysed cells. Increasing amounts of trypsin-treated microsomes derived from a control strain (YPH500(pCGS109); YEp::GAL) or a strain overproducing Sec12p (YPH500(pCEY5); YEp::Gal-SEC12), both of which were grown in galactose-containing medium, were preincubated with the cytosol and the HSP fractions for 10 min at 0°C before mixing with the membranes that contained pro-α-factor. The efficiency of transport for each reaction was compared to the maximum transport efficiency obtained when competitor membranes were omitted (25%).

Membranes were isolated from two strains varying only in their content of Sec12p. Wild-type (YPH500/pCGS109; YEp::GAL) and a Sec12p-overproducing strain (YPH500/pCEY5; YEp::GAL-SEC12) were grown on galactose to induce maximal synthesis of Sec12p in the latter. Membranes were isolated and treated with trypsin under conditions where Sec12p remains intact, but ER–Golgi transport activity is inactivated (Nakano et al., 1988; d'Enfert, C., unpublished data). Membranes from the transformant contained at least 60-fold more Sec12p than preparations from wild-type cells. Equivalent amounts of membrane protein from transformed and untransformed cells were mixed with cytosol and HSP fractions for 10 min at 0°C, and aliquots then added to a stage II incubation containing wild-type ER membranes loaded with [^{35}S]α-factor precursor. Transport inhibition related to the amount of Sec12p in the overproducer membrane fraction was observed (Fig. 2). Competitor membranes isolated from cells transformed with a multicopy plasmid containing *SEC12* under control of its own promoter (six- to eightfold less Sec12p than the GAL-regulated *SEC12*) were threefold more potent than control membranes in inhibiting transport (not shown). Inhibition was proportional to but not linearly related to the Sec12p level. Not all of the Sec12p may be in a form capable of competing for a transport-limiting component.

Sar1p Is Limiting in the Inhibited Reaction

The model presented in Fig. 1 implies that Sec12p titrates a limiting cytosolic component, however, Sec12p could equally well limit a factor present in the membrane or HSP fraction. Trypsin treatment of the Sec12p-rich membrane should reduce the contribution of a peripheral membrane protein that could partition between the cytosol and membrane fraction. Indeed, trypsin treatment enhanced (1.5-fold) the inhibitory potency of the competitor membrane fraction

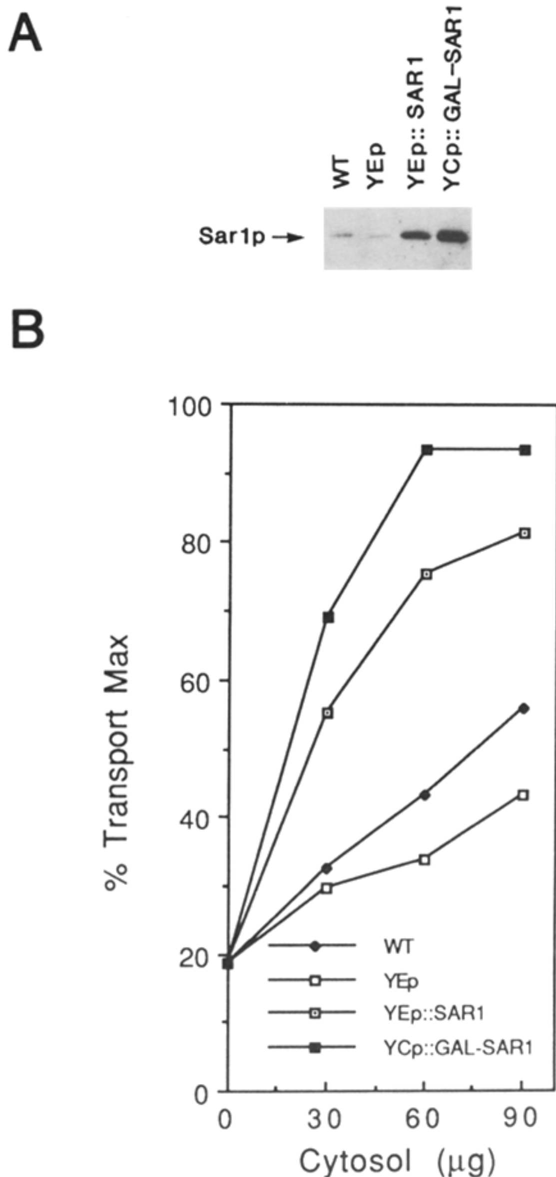

Figure 3. Cytosol-dependent reversal of Sec12p-mediated inhibition. Cytosols prepared from RSY607 (*WT*) grown in YP 2% glucose and from YPH500(pSEY8) (*YEp*), YPH500(pANY2-7) (*YEp::SAR1*), and YPH500(pANY2-18) (*YCp::GAL-SAR1*) grown in MV 2% lactate 2% galactose were compared for their Sar1p content and their effect on the Sec12p-dependent transport inhibition. (*A*) 25 μg of each cytosol was resolved by SDS-PAGE and immunoblotted with anti–Sar1p antibodies. (*B*) Standard transport reactions were carried out with wild-type membranes containing translocated pro-α-factor, 150 μg wild-type cytosol, 40 μg wild-type HSP prepared from bead-lysed cells, 5 μg trypsin-treated Sec12p-enriched microsomes, and increasing amounts of cytosol from each strain. The efficiency of transport for each reaction was compared to the maximum transport efficiency obtained when Sec12p-rich microsomes and additional cytosol were omitted (23%). Addition of Sec12p-rich microsomes alone resulted in a 81% inhibition of transport.

(not shown). Inhibition was partially overcome by addition of higher-than-normal levels of cytosol to incubations that contained the membrane mixture (Fig. 3 *B*).

Given the known genetic interaction between *sec12* and *SAR1* (Nakano and Muramatsu, 1989), we examined the effect of cytosols prepared from strains transformed either with a 2μ multicopy vector carrying *SAR1* under control of its own promoter, with a single-copy plasmid carrying *SAR1* under control of the more active *GAL1*-10 promoter, or with a control 2μ plasmid. The relative content of Sar1p in these cytosol fractions was compared by immunoblotting with a polyclonal antiserum (Fig. 3 *A*). The 2μ *SAR1* produced ∼20-fold more, and the *CEN GAL::SAR1* ∼45-fold more Sar1p than the untransformed and control transformed strains, as determined by densitometry of the SDS-PAGE immunoblot. Significantly greater rescue of transport was observed with cytosols that contained more Sar1p (Fig. 3 *B*). Although the antidote effect was proportional but not linearly related to the level of Sar1p overproduction, other factors may become limiting or not all of the Sar1p may be functional. Other genes (*SEC13*, *SEC16*) have been associated with *SEC12* by genetic analysis (Kaiser and Schekman, 1990). Cytosol fractions prepared from cells transformed with multicopy vectors carrying these genes were no more potent than control cytosol in ameliorating inhibition by Sec12p-rich membranes, although they contained increased amounts of these SEC proteins (not shown). Thus the inhibition by excess Sec12p is overcome by Sar1p or by some factor that is coordinately induced by overproduction of Sar1p.

To examine the specificity of rescue by Sar1p, cytosol from the *GAL::SAR1* transformant was fractionated by gel filtration on Sephacryl S-100HR. Sar1p filtered in the included volume with a peak of immunoreactive material in fractions 30-34 (Fig. 4 *A*). Rescue assays were performed by mixing trypsin-treated Sec12p-rich membranes with a normal dose of the cytosol and HSP fractions and aliquots of the Sephacryl column fractions. After 10 min at 0°C the mixture was added to wild-type membranes containing pro-α-factor followed by incubation in a stage II transport reaction. Rescue activity fractionated coincidentally with Sar1p and was recovered in 18% yield with respect to the starting cytosol fraction. This level of recovery is consistent with the possibility that another cytosolic factor became limiting in the presence of competitor membranes. Although Sar1p is a 21-kD protein (Nakano and Muramatsu, 1989), the polypeptide eluted from the Sephacryl column at the position expected of a 7.5-kD protein. This may account for the striking separation of Sar1p (Fig. 4 *B*, *closed circles*) from the total protein (*open circles*) on this column. Indeed, SDS-PAGE of Sar1p peak fractions of the Sephacryl column showed the protein highly enriched in relation to other polypeptides (Fig. 5). Sar1p overproduction was insufficient to detect a difference in SDS-PAGE comparison of cytosol fractions prepared from untransformed and transformed cells (not shown). Parallel Sephacryl fractionation of cytosol from a control strain grown on galactose showed little rescue activity (not shown) and Sar1p was not detected in equivalent column fractions (Fig. 5). Sar1p appears to be directly responsible for reversal of the inhibitory effect of excess Sec12p.

Sar1p Stimulates Vesicle Budding

Although Sar1p is required for protein transport from the ER (Nakano and Muramatsu, 1989), a role in vesicle budding or fusion has not been distinguished. We used a vesicle budding assay that detects the release of transport vesicles from the ER to monitor the effect of Sec12p-rich membranes and

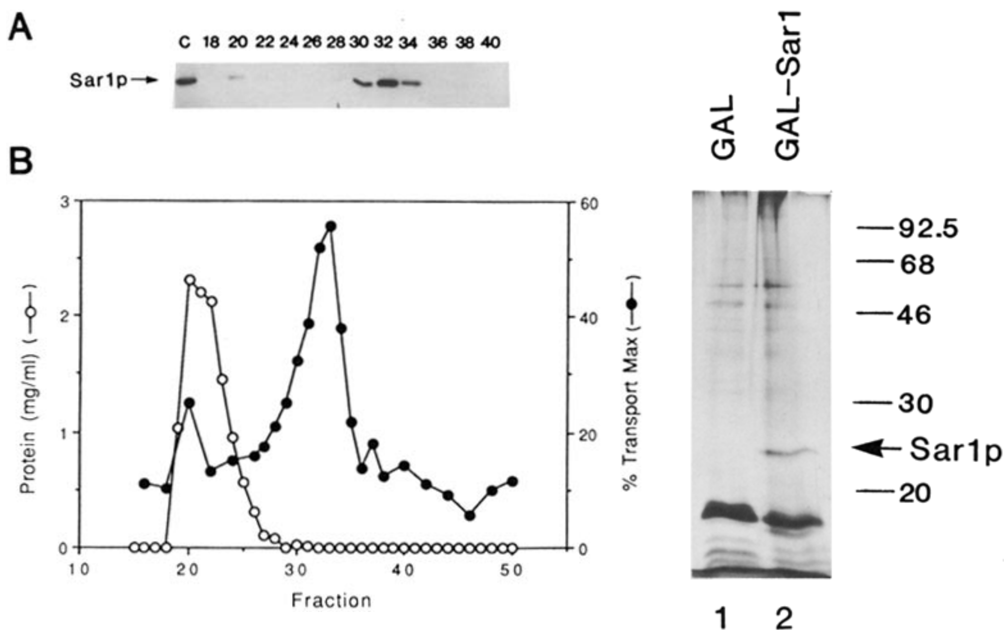

Figure 4. Sarlp copurifies with the activity that reverses transport inhibition. A cytosol derived from YPH500(pANY2-18) grown in YP 2% galactose was fractionated onto a 20-ml Sephacryl S-100 HR gel filtration column. Fractions (400 μl) were examined for Sarlp content, effect on transport inhibition, and protein concentration. (*A*) Representative fractions (8 μl) were resolved by SDS-PAGE and immunoblotted with anti-Sarlp antibodies. Unfractionated cytosol (20 μg) is shown as a control for Sarlp (*C*). (*B*) Standard transport reactions were carried out with 10 μg wild-type membranes, 150 μg wild-type cytosol, 40 μg wild-type HSP prepared from bead-lysed cells, 5 μg trypsin-treated Secl2p-enriched microsomes, and 20 μl of column fractions. The efficiency of transport for each reaction was compared to the maximum transport efficiency obtained when Secl2p-rich microsomes and column fractions were omitted (14%). Addition of Secl2p-rich microsomes alone resulted in an 88% inhibition of transport. 68% of the maximum transport efficiency was recovered when 20 μg of the unfractionated cytosol was added to an inhibited reaction. The protein concentration in each fraction is shown. The Sephacryl S-100 column was calibrated with markers that filtered to the following positions: void volume, blue dextran fraction 20; included volume, ovalbumin (43 kD) fraction 24; ribonuclease A (14 kD) fraction 30; column volume, $CoCl_2$ fraction 45–50.

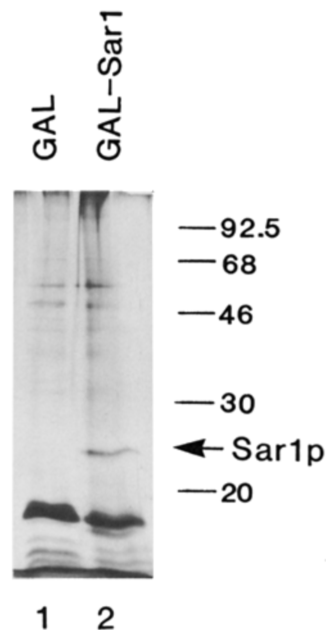

Figure 5. Sarlp enrichment by gel filtration. Cytosols prepared from control (YPH-500(pCGS109); *GAL*) and Sarlp overproducing (YPH-500(pANY2-18); *GAL-SARI*) strains grown in YP 2% galactose were fractionated on an S-100 HR gel filtration column. Fractions corresponding to the peak of rescue activity obtained with the Sarlp-enriched cytosol were resolved by SDS-PAGE followed by silver staining. The elution of Sarlp is indicated.

rescue by Sarlp. In this assay, vesicle budding from an enriched ER fraction is monitored by the appearance of protease-protected core-glycosylated [^{35}S]pro-α-factor in a slowly sedimenting compartment that is resolved from ER by differential centrifugation (Wuestehube, L., and R. S. Schekman, manuscript in preparation). Vesicle release is cytosol- and energy-dependent and is retarded by GTPγS. Released vesicles are competent for transfer of core glycosylated pro-α-factor to a fresh supply of membranes.

Secl2p-rich membranes inhibited vesicle budding (Fig. 6). Membranes isolated from the GAL-regulated *SEC12* transformant decreased budding by 69%, whereas membranes isolated from an untransformed cell decreased budding by 4%. Budding inhibition was overcome by addition of cytosol from a Sarlp overproducing strain (restored to 75% of normal), while the same amount of cytosol from an untransformed strain offered no rescue. Sephacryl S-100 enriched fractions of Sarlp also were active as an antidote to inhibition of budding by Secl2p-rich membranes (not shown). These results suggest that Sarlp interacts with Secl2p to facilitate the formation of transport vesicles from the ER.

Membrane Binding of Sarlp Is Stimulated by Secl2p

Secl2p may act to sequester Sarlp by recruitment to the membrane fraction or by inactivation of a form that remains cytosolic. Membranes containing normal or overproduced levels of Secl2p were used to examine recruitment of Sarlp from a crude cytosol or of Sarlp purified on Sephacryl S-100. Membrane fractions were treated with trypsin to remove endogenous Sarlp and mixed with crude or purified Sarlp fractions at 4°C. The content of Secl2p and the redistribution of Sarlp to membranes was evaluated by immunoblotting particulate (medium speed pellet [*MSP*]) and soluble (medium speed supernatant [*MSS*]) fractions resolved by SDS-PAGE (Fig. 7). Secl2p was detected by immunoblot only in membranes derived from cells transformed with a multicopy vector containing *SEC12* (Fig. 7, *S*, lanes *2*, *4*, *7*, and *10*); Secl2p was only partially sensitive to trypsin cleavage (compare lane *2* with lanes *4*, *7*, and *10*). Untreated membranes from the *SEC12* transformant contained more endogenous Sarlp than was found in untransformed control membranes (Fig. 7, compare lanes *1* and *2*). This species was quantitatively converted by trypsin to a Sarlp fragment (Fig. 7, *SarlΔ*) that sedimented in the MSP fraction. Exogenous Sarlp became sedimentable only when mixed with a membrane fraction (Fig. 7, compare lanes *5* and *8* with lanes *6* and *7* and *9* and *10*), and the proportion of Sarlp recruited to membranes was enhanced by elevated levels of Secl2p (three- to fivefold, compare Fig. 7, lanes *6* and *7*, and lanes *9* and *10*). Recruitment to the membrane occurred at 4°C and was independent of ATP or GTP (not shown). Although membrane association of Sarlp occurred even with membranes that contained the normal low level of Secl2p, this could reflect some non-specific binding. Purified Sarlp binds

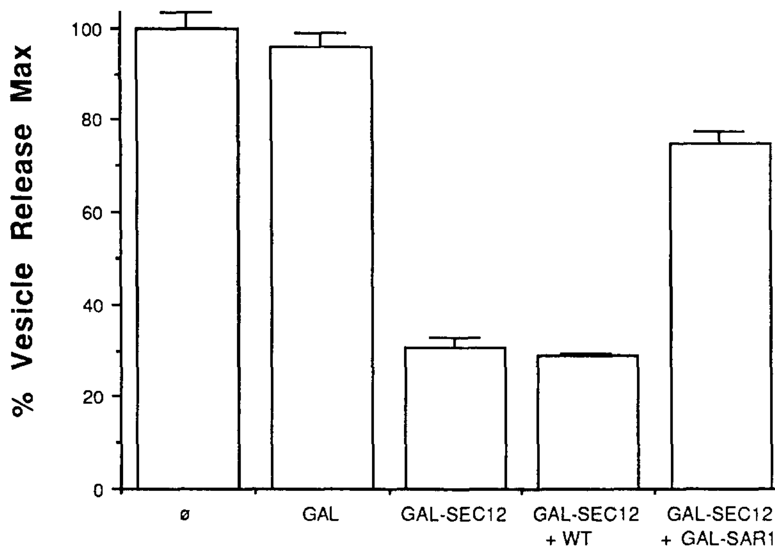

Figure 6. Sar1p requirement for the assembly of ER-derived transport vesicles. Vesicle formation assays were carried out in the presence of 470 μg of wild-type cytosol and additional fractions. (∅) no addition; GAL: 100 μg control microsomes (YPH500(pCGS-109)); (*GAL-SEC12*) 100 μg Sec12p-rich microsomes (YP500(pCEY5)); (*WT*) 125 μg wild-type cytosol (RSY607); (*GAL-SAR1*) 125 μg Sar1p-rich cytosol (YPH500(pANY2-18)). The efficiency of vesicle release for each reaction was compared to the maximum obtained when no additional fractions were added (24%). The data shown is the average value of reactions performed in triplicate and error bars represent the standard deviation.

to soybean phospholipid liposomes (Barlowe, C., unpublished results). Thus, at least part of the inhibitory effect of Sec12p-rich membranes on ER vesicle budding is due to membrane recruitment and sequestering of soluble Sar1p.

Discussion

Sar1p is a small GTP-binding protein required for efficient protein transport from the endoplasmic reticulum to the Golgi apparatus (Nakano and Muramatsu, 1989; Nishikawa and Nakano, 1991). The *SAR1* gene was isolated as a multicopy suppressor of a thermosensitive mutation in the *SEC12* gene which encodes a membrane glycoprotein involved in the formation of ER-to-Golgi transport vesicles (Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990; Rexach and Schekman, 1991). These results suggested that Sar1p could interact with the cytosolic domain of Sec12p to promote vesicle assembly. This assumption is now confirmed by our observation that Sar1p becomes limiting in a vesicle budding reaction when the level of Sec12p is elevated in a competitor membrane fraction. In addition, Sec12p-rich membranes bind Sar1p more abundantly than do normal membranes which have a low level of Sec12p. Independently, Oka et al. (1991) have shown that Sar1p isolated from transformed yeast or *Escherichia coli* suppresses temperature sensitive transport in the ER→Golgi transport reaction reconstituted with *sec12* mutant membranes. Although all these results point to an interaction between Sar1p and Sec12p, the contact may not be direct or stable, and additional factors that may not be rate-limiting could mediate the interaction.

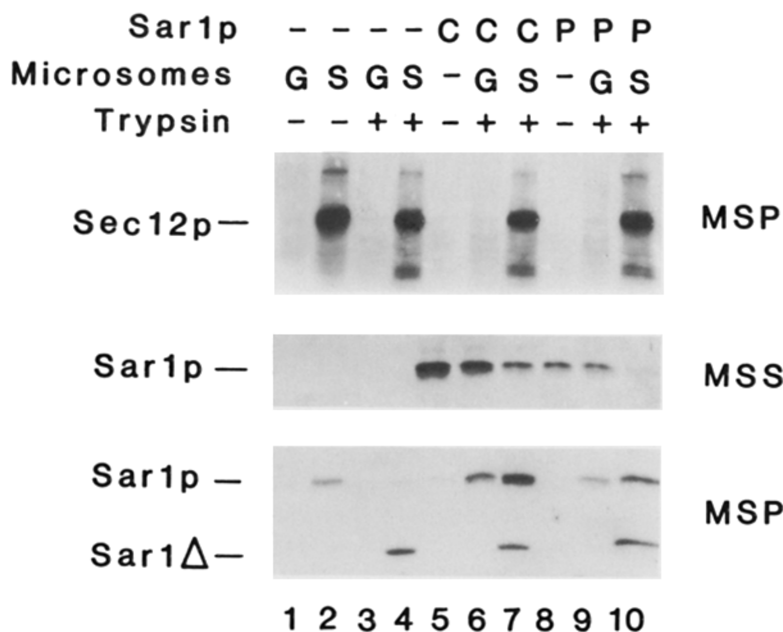

Figure 7. Sec12p-dependent membrane binding of Sar1p. Control microsomes (20 μg) (*Microsomes G*; YPH500(pCGS109)) or Sec12p-rich membranes (*Microsomes S*; YPH500(pCEY5)) were treated with trypsin (*Trypsin +*; 200 μg/ml, 30 min, 4°C). After inactivation of the protease, microsomes were mixed with either reaction buffer (*Sar1p −*), 50 μg of a Sar1p-rich cytosol (*Sar1p C*; YPH500(pANY2-18)) or 25 μl of partially purified Sar1p obtained after gel filtration of this same cytosol (*Sar1p P*). In the latter case, BSA (1 mg/ml) was included to avoid Sar1p proteolysis. Sar1p-containing fractions were also incubated in the absence of membranes (*Microsomes −*). After a 5-min incubation on ice, the soluble fraction (*MSS*) and membrane fraction (*MSP*) were separated by a 25,000 *g* centrifugation and their content analyzed by SDS-PAGE and immunoblotting with anti-Sar1p or anti-Sec12p antibodies.

Sar1p Requirement for the Formation of ER to Golgi Transport Vesicles

Apart from *SAR1*, genetic analysis had identified three other genes, *SEC4*, *YPT1*, and *ARF1*, that encode small GTP-binding proteins required for protein transport at different steps along the secretory pathway (Salminen and Novick, 1987; Segev et al., 1988; Stearns et al., 1990). In the case of Sec4p, localization of the protein as well as mutational analysis suggest that it is required for proper targeting and fusion of secretory vesicles to the plasma membrane (Goud et al., 1988; Walworth et al., 1989). Biochemical studies have shown that ER-to-Golgi transport and *cis*-to-medial Golgi transport is inhibited by GTPγS (Malençon et al., 1987; Baker et al., 1988; Ruohola et al., 1988; Beckers and Balch, 1989) and by synthetic peptides corresponding to the effector domain of members of the *rab* family of mammalian Sec4p/Ypt1p-related proteins (Plütner et al., 1990; Zaharoui et al., 1989). In mammalian cells, GTPγS inhibition results in the accumulation of intra-Golgi non-clathrin–coated–vesicles docked to their acceptor compartment (Melançon et al., 1987; Orci et al., 1989; Malhotra et al., 1989). In yeast, Ypt1p is required for efficient in vitro ER to Golgi transport (Baker et al., 1990; Bacon et al., 1989) and its immobilization by Ypt1p antibody Fab fragments results in the accumulation of transport vesicles en route to the Golgi (Rexach and Schekman, 1991). Bourne (1988) has proposed a unifying model which suggests that each GTP-binding protein is required at a specific step to ensure an efficient targeting of vesicular carriers to their acceptor compartment.

It appears that GTPγS also inhibits vesicle budding or release from the *trans*-Golgi network (Tooze et al., 1990) and from the ER (Rexach and Schekman, 1991; Wuestehube, L., and R. Schekman, manuscript in preparation). We have shown that Sar1p is required for vesicle assembly during ER-Golgi transport and is thus the likely target of GTPγS inhibition in this process. A model for the function of Sar1p in vesicle formation is proposed in Fig. 8. Together with the involvement of Ypt1p in ER-Golgi transport (Segev et al., 1988; Baker et al., 1990; Rexach and Schekman, 1991), our results suggest that Sar1p and Ypt1p may function in successive steps using GTP binding and hydrolysis to facilitate protein transport from the ER.

Structural differences between Sar1p and Ypt1p support the view that they act at different stages during a single round of vesicular traffic. Whereas Ypt1p is closely related to the mammalian oncogene ras (Gallwitz et al., 1982), the Sar1p nucleotide binding site (G-1 and G-3 regions; Bourne et al., 1991) is more closely related to the family of ADP-ribosylation factors (ARF; Kahn, 1990; Nakano and Muramatsu, 1989; Bourne et al., 1991). ARF is associated with intra-Golgi transport vesicles in mammalian cells (Serafini et al., 1990) and its yeast homologue, Arf1p, is required for protein transport and also is localized to the Golgi apparatus (Sewell and Kahn, 1988; Stearns et al., 1990). ARF could play a role similar to that of Sar1p but at a later stage in secretion.

Sec12p-dependent Membrane Attachment of Sar1p

A functional difference between Ypt1p and Sar1p is also suggested by different possible mechanisms of membrane attachment. Ypt1p, Sec4p, and members of the *rab* family of small GTP-binding proteins (Haubrück et al., 1987; Touchot et al., 1987; Zahraoui et al., 1989; Chavrier et al., 1990) have at least one cysteine that is located near the COOH terminus of the protein. By analogy with the mammalian *ras* oncogene (Hancock et al., 1989), this residue is thought to be modified by a lipid, possibly a farnesyl analogue, which mediates membrane attachment (Molenaar et al., 1988; Walworth et al., 1990). Sar1p does not have a carboxy-terminal cysteine nor any site of putative lipid modification that could explain its strong affinity for membranes (Nishikawa and Nakano, 1991). In this study, we have established an in vitro assay that measures the binding of Sar1p to ER membranes. Results presented in Fig. 7 show that Sar1p membrane attachment is stimulated by elevated levels of Sec12p. Thus, Sar1p may become membrane bound through the intervention of Sec12p.

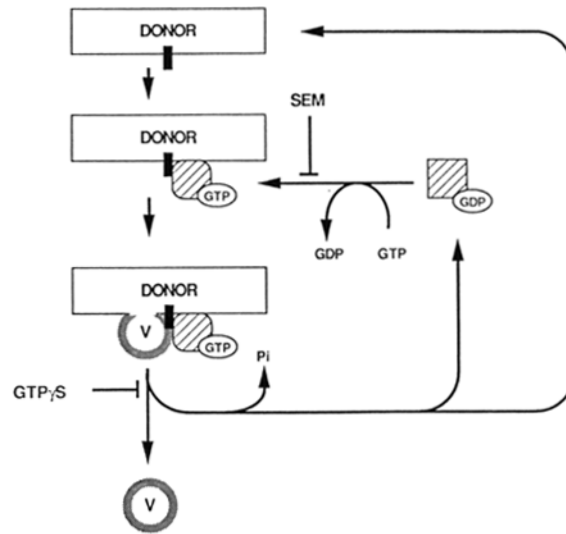

Figure 8. A diagram for the function of Sar1p and Sec12p in the formation of ER-derived vesicles. Attachment of Sar1p (*hatched box*) to the membrane is mediated by Sec12p (*solid rectangle*). The blocks imposed by GTPγS and by an excess of Sec12p-enriched membranes (*SEM*) in vesicle release are shown.

Sec12p could act as a membrane anchor for Sar1p or alter the conformation of Sar1p so as to expose a hydrophobic surface enabling direct contact with the lipid bilayer. In both cases, Sec12p-specific membrane attachment of Sar1p could result in the regulated formation of an ER-Golgi transport vesicle at specific sites on the ER membrane. Studies with purified forms of both proteins will distinguish these possibilities.

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References

Bacon, R., A. Salminen, H. Ruohola, P. Novick, and S. Ferro-Novick. 1989. The GTP-binding protein Ypt1 is required for transport *in vitro*: the Golgi apparatus is defective in *ypt1* mutants. *J. Cell Biol.* 109:1015–1022.

Baker, D., L. Hicke, M. Rexach, M. Schleyer, and R. Schekman. 1988. Recon-

stitution of *SEC* gene product-dependent intercompartmental protein transport. *Cell.* 54:335–344.

Baker, D., L. Wuestehube, R. Schekman, D. Botstein, and N. Segev. 1990. GTP-binding Ypt1 protein and Ca^{2+} function independently in a cell-free protein transport reaction. *Proc. Natl. Acad. Sci. USA.* 87:355–359.

Beckers, C., and W. Balch. 1989. Calcium and GTP: essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. *J. Cell Biol.* 108:1245–1256.

Bourne, H. R. 1988. Do GTPases direct membrane traffic in secretion? *Cell.* 53:669–671.

Bourne, H. R., D. A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature (Lond.).* 349: 117–127.

Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low-molecular weight GTP-binding proteins to exocyotic and endocytic compartments. *Cell.* 62:317–329.

Emr, S. D., A. Vassarotti, J. Garrett, B. L. Geller, M. Takeda, and M. G. Douglas. 1986. The aminoterminus of the yeast F_1-ATPase β-subunit precursor functions as a mitochondrial import signal. *J. Cell Biol.* 102:523–533.

Gallwitz, D., C. Donath, and C. Sander. 1983. A yeast gene encoding a protein homologous to the human C-has/bas proto-oncogene product. *Nature (Lond.).* 306:704–707.

Goud, B., A. Salminen, N. C. Walworth, and P. Novick. 1988. A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. *Cell.* 53:753–768.

Hancock, J. H., A. I. Magee, J. E. Childs, and C. J. Marshall. 1989. All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell.* 57:1167–1177.

Haubrück, H., C. Disela, P. Wagner, and D. Gallwitz. 1987. The *ras*-related ypt protein is an ubiquitous eukaryotic protein: isolation and sequence analysis of mouse cDNA clones highly homologous to the yeast *YPT1* gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:4049–4053.

Hicke, L., and R. Schekman. 1989. Yeast Sec23p acts in the cytoplasm to promote protein transport from the endoplasmic reticulum to the Golgi complex *in vivo* and *in vitro*. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1677–1684.

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

Kahn, R. 1990. The ADP-ribosylation factor of adenylate cyclase: a 21 kDa GTP-binding protein. *In* G-proteins. L. Birbaumer and R. Lyengar, editors. Academic Press, Inc., Orlando, FL. 201–204.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Malhotra, V., T. Serafini, L. Orci, J. C. Shepherd, and J. E. Rothman. 1989. Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell.* 58:329–336.

Melançon, P., B. S. Glick, V. Malhotra, P. J. Weidman, T. Serafini, M. L. Gleason, L. Orci, and J. E. Rothman. 1987. Involvement of GTP-binding "G" proteins in transport through the Golgi stack. *Cell.* 51:1053–1062.

Molenaar, C. M. T., R. Prange, and D. Gallwitz. 1988. A carboxyterminal cysteine residue is required for palmitic acid binding and biological activity of the *ras*-related YPT1 protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:971–976.

Morrisey, J. H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* 117:307–310.

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.

Nakano, A., D. Brada, and R. Schekman. 1988. A membrane glycoprotein, Sec12p, required for protein transport from the endoplasmic reculum to the Golgi apparatus and yeast. *J. Cell Biol.* 107:851–863.

Nishikawa, S., and A. Nakano. 1991. The GTP-binding Sar1 protein is localized to the early compartment of the yeast secretory pathway. *Biochem. Biophys. Acta.* In press.

Oka, T., S. Nishikawa, and A. Nakano. 1991. Reconstitution of GTP-binding Sar1 protein function in ER to Golgi transport. *J. Cell Biol.* 114:671–679.

Orci, L., V. Malhotra, M. Amherdt, T. Serafini, and J. E. Rothman. 1989. Dissection of a single round of vesicular transport: sequential intermediates for intercisternal movement in the Golgi stack. *Cell.* 56:357–378.

Plütner, H., R. Schwaninger, S. Pind, and W. E. Balch. 1990. Synthetic peptides of the *Rab* effector domain inhibit vesicular transport through the secretory pathway. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2375–2383.

Rexach, M., and R. Schekman. 1991. Distinct biochemical requirements for the budding, targeting, and fusion of ER-derived transport vesicles. *J. Cell Biol.* 114:219–229.

Rose, M. D., L. M. Misra, and J. P. Vogel. 1989. *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/*GRP78* gene. *Cell.* 57:1211–1221.

Ruohola, H., A. K. Kabcenell, and S. Ferro-Novick. 1988. Reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex in yeast: the acceptor Golgi compartment is defective in the *sec23* mutant. *J. Cell Biol.* 107:1465–1476.

Salminen, A., and P. J. Novick. 1988. A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell.* 49:527–538.

Schekman, R. 1985. Protein localization and membrane traffic in yeast. *Annu. Rev. Cell Biol.* 1:115–143.

Segev, N., J. Mulholland, and D. Botstein. 1988. The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. *Cell.* 52:915–924.

Serafini, T., M. Brunner, R. A. Kahn, and J. E. Rothman. 1990. ADP-ribosylation factor (ARF) is a constituent of the non-clathrin-coated transport vesicles. *J. Cell. Biochem.* 14C:64.

Sewell, J. L., and R. A. Kahn. 1988. Sequences of the bovine and yeast ADP-ribosylation factor and comparison to other GTP-binding proteins. *Proc. Natl. Acad. Sci. USA.* 85:4620–4624.

Sikorski, R. S., and P. Hieter. 1989. A system of shuffle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics.* 122:19–27.

Stearns, T., M. C. Willingham, D. Botstein, and R. A. Kahn. 1990. ADP-ribosylation factor is functionally and physically associated with the Golgi complex. *Proc. Natl. Acad. Sci. USA.* 87:1238–1242.

Tooze, S. A., U. Weiss, and W. B. Hüttner. 1990. Requirement for GTP hydrolysis in the formation of secretory vesicles. *Nature (Lond.).* 347:207–208.

Touchot, N., P. Chardin, and A. Tavitian. 1987. Four additional members of the *ras* gene superfamily isolated by an oligonucleotide strategy: molecular cloning of YPT-related cDNAs from a rat brain library. *Proc. Natl. Acad. Sci. USA.* 84:8210–8214.

Towbin, M., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4353.

Walworth, N. C., B. Goud, A. Pastan Kabcenell, and P. Novick. 1989. Mutational analysis of *SEC4* suggests a cyclical mechanism for the regulation of vesicular traffic. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1685–1693.

Zahraoui, A., N. Touchot, P. Chardin, and A. Tavitian. 1989. The human *Rab* genes encode a family of GTP-binding proteins related to the yeast YPT1 and SEC4 products involved in secretion. *J. Biol. Chem.* 264:12394–12401.