Purification and Characterization of SAR1p, a Small GTP-binding Protein Required for Transport Vesicle Formation from the Endoplasmic Reticulum*

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SEC12 encodes an integral membrane glycoprotein essential for vesicle formation from the endoplasmic reticulum (ER) in yeast. The SAR1 gene was discovered as a multicopy suppressor of a sec12 strain and encodes a 21-kDa GTP-binding protein also required for protein transport from the ER to the Golgi apparatus (Nakano, A., and Muramatsu, M. (1989) J. Cell Biol. 109, 2677–2681). We have purified Sar1p to apparent homogeneity from cells harboring a galactose-regulated recombinant SAR1. Purified Sar1p binds guanine nucleotides specifically and exhibits GTPase activity (0.001 min	extsuperscript{−1}). Nucleotide exchange and hydrolysis rates are greatly increased in the presence of Mg	extsuperscript{2+} and nonionic detergents or phospholipids. An assay that measures the formation of a vesicle intermediate in ER to Golgi transport was devised that is dependent on the addition of purified Sar1p. This assay employs membranes prepared from wild-type cells and cytosol fractions depleted of Sar1p due to overproduction of Sec12p or by gel filtration chromatography. The gel-filtered cytosol requires the addition of Sar1p and GTP to support vesicle budding. Sar1p prebound with GTP	extsuperscript{γ}S inhibits Sar1p function in the vesicle formation assay. The results indicate a role for Sar1p in vesicle budding from the ER and suggest that GTP hydrolysis by Sar1p is required for this event.

The secretory pathway in eukaryotic cells transports proteins destined to organelles and the cell surface through a series of membrane-bound compartments. An early step in the secretory pathway is the formation of a vesicle intermediate from the endoplasmic reticulum (ER) that is then transported to the Golgi apparatus (Palade, 1975). Through a combined genetic and biochemical approach, our laboratory investigates transport vesicle formation from the ER in the yeast Saccharomyces cerevisiae. Genetic studies have implicated SEC12, 13, 16, 23, and SAR1 in vesicle formation from the ER (Novick et al., 1980; Kaiser and Schekman, 1990; Nakano and Muramatsu, 1989). Cell-free assays in yeast have been developed that reconstitute vesicle formation from the ER and protein transport to the Golgi apparatus in cytosol and ATP-dependent reactions (Baker et al., 1988; Ruohola et al., 1988; Groesch et al. 1990; Rexach and Schekman, 1991). The cell-free assay has corroborated genetic observations, implicating Sec12p, Sec23p, and Sar1p function in vesicle formation (Rexach and Schekman, 1991; d’Enfert et al., 1991a). An additional protein, p105 (Sec24p), that copurifies with functional Sec23p, is also essential for vesicle formation in vitro (Hicke et al., 1992). As a step toward our goal of reconstituting vesicle formation with ER membranes and purified cytosolic components, we report the purification and characterization of Sar1p, a small GTPase required for vesicle formation from the ER.

SAR1 was discovered as a multicopy suppressor of a temperature-sensitive sec12 strain and encodes a protein with sequence homology to the ras superfamily of small GTPases (Nakano and Muramatsu, 1989). Sar1p is a novel member of this family with highest amino acid identity to yeast ARF1 (35% identity over 168 residues; Nakano and Muramatsu, 1989). The temperature-sensitive transport defect of sec12, which is reproduced in the in vitro transport assay (Rexach and Schekman, 1991), can be repaired at the restrictive temperature by adding excess Sar1p (Oka et al., 1991). Sar1p has been proposed to function in the vesicle formation step of ER to Golgi transport (d’Enfert et al., 1991a). Several other small GTPases have been identified in the yeast secretory pathway including Ypt1p, Arf1p, and Sec4p (Segev et al., 1988; Stearns et al., 1990; Salminen and Novick, 1987). Although the importance of small GTPases in the secretory pathway is well appreciated, a mechanism for GTPase function in vesicle formation or targeting is obscure. Thus, in vitro reconstitution of ER vesicle formation with purified and characterized components will allow us to examine one event that is likely to be a paradigm for organelar budding.

EXPERIMENTAL PROCEDURES

Radiolabeled nucleotides, [α-32P]GTP, [γ-32P]GTP, and [3H]GDP were purchased form Amersham Corp. [35S]GTPγS was obtained from Du Pont-New England Nuclear. Soybean phospholipids (45% phosphatidyl choline extract) were supplied by Avanti Polar Lipids (Alabaster, AL). Yeast strains used in this study were RSY807 (MATa ura3-52 lys2-801 ade2-101 trpl-1 ade2-1 [trpl-1 363 his3·Δ200 leu2-3112]) containing plasmid pANY1-9 (2 μm URA3 SEC12) (Nakano et al., 1988) was obtained after sporulation of strain RSY656 (d’Enfert et al., 1991b) transformed with pANY1-9. Plasmid pANY2-18 (CEN4-ARS1 TRP1 GAL1-SAR1) was de-
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Sarlp Purification—Two liters of strain YPH500 harboring plasmid pANY2-18 (GALI-SAR1) was grown at 30 °C to late log phase in 3% yeast extract supplemented with appropriate amino acids and 2% raffinose. Cells were harvested by centrifugation and resuspended in 4 liters of 1% yeast extract, 2% peptone, and 2% galactose to induce expression from the GALI promoter. After growth at 30 °C for 14 h, cells (approximately 20,000 O.D.600 nm) were harvested and washed once with ice-cold buffer 88 (0.25 M sorbitol, 20 mM K-HEPES, pH 6.9, 1 mM MgOAc2, and 150 mM KOAc, pH 6.8). The following steps of the purification were performed at 4 °C. The cell pellet was resuspended in 60 ml of buffer 88 supplemented with 1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol and lysed in a Ball-beater chamber (Biospec Products, Bartlesville, OK) containing 1/5 volume of glass beads, by six 1-min periods of agitation. The lysate was decanted, and the glass beads were washed with 20 ml of buffer 88 to increase recovery. A combined crude extract was centrifuged at 20,000 × g for 20 min, and the resulting supernatant fluid was clarified by centrifugation at 100,000 × g for 60 min in a Beckman Ti45 rotor. Typically, 60 ml of this high speed supernatant material was recovered and divided into five 1-ml aliquots. One of the 1-ml aliquots was kept in liquid N2 for storage at -70 °C. Each of the 20-ml aliquots was thawed on ice and then incubated at 29 °C for 1 h. One ml of each 20-ml aliquot was washed with 50 ml of equilibration buffer and then 100 ml of equilibration buffer was added to a final concentration of 0.02% Triton X-100 and frozen in liquid NP for storage at -70 °C.

Fractions were collected. Sarlp, as determined by a Sarlp-dependent vesicle budding assay (d’Enfert et al., 1991a), was eluted with 20 mM K-HEPES (pH 6.8), 200 mM KOAc, 1 mM MgOAc2, and 0.02% Triton X-100 to reduce the KOAc concentration to 37.5 mM and loaded onto an 8-ml, 1 ml affinity column of Sarlp-Sepharose 4B (Pharmacia LKB Biotechnology Inc.) equilibrated with buffer 88. Protein was eluted with buffer 88 at a flow rate of 1 ml/min, and 8-ml fractions were collected. Sarlp, as determined by a Sarlp-dependent vesicle budding assay, was described as specific immunoreactivity, eluted as a broad peak at a fraction corresponding to 7 kDa and was clearly resolved from the majority of protein (d’Enfert et al., 1991a). A pool of the peak Sarlp-containing fractions (45 ml) was adjusted to a final concentration of 0.02% Triton X-100 and frozen in liquid N2 for storage at -70 °C. Three S-100 pools were combined and diluted 4-fold with 0.02% Triton X-100 to reduce the KOAc concentration to 37.5 mM and loaded onto an 8-ml, 1 × 5-cm DEAE-Sepharose CL-4B (Pharmacia) column equilibrated with 20 mM K-HEPES (pH 6.8), 20 mM KOAc, 1 mM MgOAc2, and 0.02% Triton X-100. The DEAE column, operated at a flow rate of 0.5 ml/min, was washed with 50 ml of equilibration buffer and then 100 ml of equilibration buffer with 90 mM KOAc and 0.006% Triton X-100. Sarlp was eluted with 20 mM K-HEPES (pH 6.8), 200 mM KOAc, 1 mM MgOAc2, and 0.001% Triton X-100. One-milliliter fractions were collected, and the peak six fractions, as judged by silver stain, were pooled, distributed into aliquots, and frozen in liquid nitrogen for storage at -70 °C. Sarlp was active for guanine nucleotide binding and vesicle formation after several months of storage at -70 °C. Storage of protein at 4 °C resulted in a 50% loss of vesicle budding activity after 1 week. Protein could be thawed and refrozen once without detectable loss of nucleotide binding or vesicle budding activity.

Guanine Nucleotide-binding Assays—Guanine nucleotide binding to Sarlp was quantitated by a filter binding method (Northup et al., 1982). The binding reactions contained 0.1% Triton X-100, 0.5 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, 1.0 mM MgOAc2, 25 mM K-HEPES (pH 6.8), 1-2 μM guanine nucleotide (10,000 dpm/μmol), and 1-10 pmol of Sarlp in a total volume of 40 μl. Reactions were mixed on ice and then incubated at 29 °C for the times indicated in the figure legends. Reactions were stopped by dilution with 800 μl of ice-cold wash buffer (0.002 M Triton X-100, 25 mM K-HEPES [pH 6.8], 100 mM NaCl, 5 mM MgCl2) containing 10 μg/ml of nitrocellulose membranes, 0.45 μM α-[32P]GTP, and 1 μM GDP. The reaction mixtures were placed on nitrocellulose membranes, washed four times with 2 ml of wash buffer, washed, and then developed with 1M LiCl, 1M HCOOH (Wagner et al., 1987). The plate was dried and conversion to GDP over time was quantitated after exposure to a Phosphorimager plate (Molecular Dynamics, Sunnyvale, CA).

Sarlp Assay in Microsome-based Vesicle Formation Reaction—Wild-type microsomes and cytosol were prepared from strain RSY607 (Wuestehube and Schekman, 1992) while a Sarlp-depleted cytosol was prepared from the Secl2p overproducer strain (d’Enfert et al., 1991a). The two-stage vesicle formation assay was performed as previously described (d’Enfert et al., 1991b). Briefly, in the stage I reaction at 10 °C, 32P-prepro-α-factor is translocated into microsomes where three N-linked core-glycosylated chains are attached yielding core-glycosylated pre-α-factor. Microsomes are then washed to remove untranslocated label and resuspended under various reaction conditions described in the figure legends and incubated at 29 °C in a total volume of 50 μl. Reactions were stopped by placing tubes on ice for 5 min then centrifuged at 12,000 × g at 4 °C for 5 min. Vesicles formed from microsomes remain in the supernatant fraction after centrifugation. The percent vesicle formation is quantified by Con A precipitation of protease-protected, core-glycosylated, pre-α-factor contained in the supernatant divided by the total protease-protected core-glycosylated pre-α-factor contained in a total reaction. A unit of Sarlp-dependent vesicle budding activity is defined as restoration of one-half maximal budding activity, eluted as a broad peak at a fraction corresponding to 7 kDa and was clearly resolved from the majority of protein (d’Enfert et al., 1991a). A pool of the peak Sarlp-containing fractions (45 ml) was adjusted to a final concentration of 0.02% Triton X-100 and frozen in liquid N2 for storage at -70 °C (Terminius of glutathione S-transferase activity). For immunoblot analysis, the fusion protein was purified on a glutathione column according to the manufacturer’s specifications (Pharmacia). Glutathione S-transferase-Sarlp fusion protein was used as antigen to raise polyclonal antiserum in rabbits. Rabbits were immunized with subcutaneous injection with 150 μg of protein in Freund’s complete adjuvant and boosted every 4 weeks with 100 μg of protein in incomplete adjuvant. Sarlp antiserum was used at a 1/2000 dilution for development of immunoblots by the enhanced chemiluminescence method (Amersham Corp.). Glutathione S-transferase-Sarlp (2 mg) was covalently linked to 1 ml of Affigel-agarose (Bio-Rad) as described by the manufacturer. This column was used to purify anti-Sarlp antibodies from serum by binding in Tris-buffered saline and elution with 0.2 M glycine-HCl, pH 2.2 (Harlow and Lane, 1988). Fab fragments from affinity-purified Sarlp antibodies were isolated after treatment with immobilized papain (Pierce Chemical Co.) as described by the manufacturer.

Other Methods—Sarlp depleted and desalted cytosol was prepared by centrifugation of a 10,000× g supernatant (6 ml) prepared from strain RSY607 on a 200-ml (2.5 cm × 45 cm) Sephacyr-S100 column. The column was eluted with buffer 88, and 4-ml fractions were collected. The first six protein-containing fractions were pooled, concentrated 2-fold with a centriprep 10 concentrator (Amicon, Beverly, MA), and stored at -70 °C. SDS-PAGE (Laemmli, 1970). Protein silver staining was performed as described by Morrisey (1981). For immunoblot, proteins were transferred to nitrocellulose (Towbin et al., 1979) and antigen detected by the enhanced chemiluminescence method (Amersham Corp.). Protein concentrations were determined (Bradford, 1976) with immunoglobulin as the standard.

RESULTS

Functional Assay for Sarlp in Vesicle Formation—Sarlp is proposed to function in vesicle formation from the ER (d’Enfert et al., 1991a). We sought to purify active Sarlp and demonstrate function in a vesicle formation reaction. An initial assay that requires Sarlp for vesicle formation was performed (d’Enfert et al., 1991b) while a Sarlp-depleted cytosol (OPC) was unable to support vesicle formation in vitro unless supplemented with Sarlp, thus allowing an assay for the purification of a functional form of Sarlp (Fig. 1). SAR1 overexpression

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The text appears to be a scientific research paper discussing the purification and characterization of Sarlp, a protein involved in vesicle formation. The methods and results are described in detail, including purification steps, vesicle formation assays, and characterization of the protein's activity. The paper references various assays and methods used for purification and analysis, indicating a thorough approach to understanding the role of Sarlp in cellular processes.
under the GAL1 promoter resulted in a ~50-fold increase in cellular Sarlp. In a wild-type strain, 80% of the Sarlp is membrane-bound; however, overexpression increases the concentration of soluble Sarlp contained in a 100,000 g supernatant (d’Enfert et al., 1991a, Nishikawa and Nakano 1991). Fractionation of the 100,000 g supernatant material on a Sephacryl S-100 gel filtration column enriched Sarlp budding activity as it eluted at the position of 7-kDa proteins and was resolved from a majority of other proteins contained in a 100,000 g supernatant (Fig. 2 and Table I). This unusual elution position suggests an interaction of Sarlp with the gel filtration matrix, and, indeed, this property results in difficulties manipulating Sarlp; we have found that this protein binds to plastic surfaces and column resins unless detergent is added. Therefore, further purification required the presence of Triton X-100. Contaminating proteins in the S-100 pool (Fig. 2, lane 4) are resolved by DEAE chromatography. Sarlp eluted from the DEAE column at 175 mM KOAc. Sarlp immunoreactivity and rescue activity coincided with the sin- gle protein that eluted from the DEAE column (not shown). SDS-PAGE demonstrated the purity of Sarlp at each step (Fig. 2), and Sarlp-specific activity in the budding assay is shown in Table I. A low yield of Sarlp was obtained, probably due to losses through hydrophobic interactions.

Sarlp purified to homogeneity restored vesicle formation activity to a Sec12p OPC (Fig. 3A, column 6). The addition of purified Sarlp to a saturating amount of WTC did not affect the budding efficiency (data not shown). Sec12p OPC supplemented with Sarlp exhibited similar but not identical kinetics to a cytosol prepared from a wild-type strain (Fig. 3B). The apparent lag in vesicle formation may reflect a requirement for Sarlp assembly into a complex prior to promoting vesicle formation.

Anti-Sarlp Antibodies Inhibit Vesicle Formation—As an independent verification of Sarlp function in vesicle formation, we tested the effect of Sarlp antibodies on a wild-type vesicle formation reaction. Potent inhibition of the vesicle formation reaction was observed with 0.5 μg of affinity-puri- fied anti-Sarlp antibodies. The divalent nature of antibodies could interfere with the vesicle formation reaction by cross-linking vesicles to the ER membranes. Thus, anti-Sarlp Fab fragments were generated and tested for inhibition. A titration curve is shown in Fig. 4 where complete inhibition was achieved by adding 0.1 μg of affinity-purified anti-Sarlp Fabs. Addition of 0.2 μg of purified yeast Sarlp relieved the antibody block at antibody concentrations below 0.2 μg.

Binding Properties—Sarlp expressed in Escherichia coli binds GTP after SDS-PAGE and renaturation on nitrocellulose (Oka et al., 1991). We determined the guanine nucleotide-binding properties by an assay in which nucleotide bound to Sarlp in solution is retained on a nitrocellulose filter. As shown in Table II, binding requires Mg2+ (maximal concentration is at 0.1 mM Mg2+) and detergent; however, the detergent requirement may be replaced by soybean phospholipids. Triton X-100 (CMC = 0.016%) and β-octylglucoside (CMC = 0.7%) were equivalent and were required at concentrations above their CMC. A somewhat related situation has been reported for mammalian ARF which requires both detergent and phospholipids to display nucleotide binding (Weiss et al., 1989). The rate of guanine nucleotide binding by Sarlp was rapid and essentially complete after 20 min at 29 °C while the binding rate at 4 °C was markedly slower (Fig. 5A). This binding rate probably represents nucleotide exchange of

![Fig. 1. Purification of Sarlp from yeast. A. silver-stained SDS-PAGE (12.5%) and B, anti-Sarlp immunoblot of material from Sarlp purification steps. Lane 1, a 100,000 g supernatant fraction (4 μg) from a lysate of strain YPH500. Lane 2, a 20,000 g supernatant fraction (4 μg) from a lysate of strain YPH500 transformed with pANY2-18 (GAL1-SARI). Lane 3, a 100,000 g supernatant fraction (4 μg) from a lysate of strain YPH500 transformed with pANY2-18. Lane 4, pool of fractions (0.2 μg) containing Sarlp after Sephacryl S-100 chromatography. Lane 5, pool of Sarlp containing fractions (0.2 μg) that eluted from the DEAE-Sepharose column.](image)
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y-globulin as a protein standard may not accurately reflect the true Sar1p protein concentration. The off rates of pre-bound GDP, GTP, and GTPγS from Sar1p were determined by the filter binding assay (Fig. 5B). The apparent first order dissociation rates are 0.071 min⁻¹, 0.091 min⁻¹, and 0.043⁻¹ for GDP, GTP, and GTPγS, respectively.

GTPase Activity of Sar1p—The rate of GTP hydrolysis by Sar1p was determined under conditions of maximal binding. Sar1p added to reactions is expressed in moles of nucleotide-binding competent protein, and [α-32P]GDP was quantified
after separation of GDP from GTP by thin layer chromatography (Fig. 6A). Production of [\(\alpha^{32}\)P]GDP was linear with time up to 90 min (Fig. 6B) and inhibited by the addition of 10 mM EDTA or a 100-fold excess of unlabeled GTP. The addition of a 100-fold molar excess of ATP had a minimal effect on GTP hydrolysis consistent with specificity for guanine nucleotides by Sarlp. A hydrolysis rate of 0.0011 min\(^{-1}\) (mol GTP hydrolyzed/mol Sarlp/min) was calculated from a time course (Fig. 6B). Production of GDP under these conditions in the absence of Sarlp was undetectable after 90 min (data not shown).

Sarlp-GTPyS Is Less Active in Supporting Vesicle Formation—We found that Sarlp bound GTP or GTPyS in the presence of soybean phospholipids (see Table II). Therefore, we addressed whether these prebound forms of Sarlp were equally capable of supporting vesicle formation. Fig. 7 shows that Sarlp-GTPyS was reduced in its ability to support vesicle formation compared to a cytosol supplemented with Sarlp-GTP. A control shown in Fig. 7A, column 5, demonstrated that phospholipids and free GTPyS (0.2 \(\mu M\)) did not affect the degree of vesicle formation with a wild-type cytosol. This control was necessary because we were unable to separate efficiently free guanine nucleotide from Sarlp-bound nucleotide used in these reactions. A time course of vesicle formation with Sarlp-GTP and Sarlp-GTPyS is shown in Fig. 7B. At early times, Sarlp-GTPyS was unable to support vesicle formation; however, at later time points, vesicle formation was apparent but about one-half that of Sarlp-GTP. This lag was likely due to an inability of Sarlp-GTPyS to support vesicle formation. Nucleotide exchange of GTPyS for GTP present in the Sec12p OPC may permit Sarlp function at later times.

Depletion of Sarlp and GTP from a wild-type cytosol by gel filtration on a Sephacryl S-100 column revealed a complete dependence on GTP hydrolysis in vesicle formation (Fig. 8). Sarlp-GTPyS was devoid of budding activity with this cytosol while Sarlp-GTP was active. GDP substituted for GTP; however, GDP\(_3\)S, a nucleotide that is not efficiently phosphorylated, was unable to support vesicle formation. These results suggest that GTP hydrolysis by Sarlp is required for vesicle budding. GDP may substitute only by virtue of conversion to GTP by diphosphonucleotide kinase and the ATP and ATP regeneration system provided to stimulate vesicle budding.

**DISCUSSION**

We have purified Sarlp and characterized its nucleotide binding, exchange, and hydrolysis properties. Further, we demonstrated that the purified form functions in an in vitro vesicle formation assay. A comparison of Sarlp binding and hydrolysis properties with other purified GTPases reveals similarities and several significant differences. Common among GTPases is the ability to bind and hydrolyze GTP in a \(Mg^{++}\)-dependent manner. Unlike most small GTPases, Sarlp has a requirement for detergent or phospholipids to bind guanine nucleotide. Interestingly, mammalian ARFs
have a similar requirement for both detergent and phospholipids for maximal nucleotide binding (Kahn et al., 1988; Weiss et al., 1989). The reason(s) for this requirement are not known but may reflect the interaction of these proteins with intracellular membranes (Nishikawa and Nakano, 1991; Serafini et al., 1991; Donaldson et al., 1991).

Sar1p contains an intrinsic GTPase (0.0011 min⁻¹) comparable to other purified small GTPases: N-ras, 0.015 min⁻¹; Ypt1p, 0.006 min⁻¹; Sec4p, 0.0012 min⁻¹ (Trahey et al., 1987; Wagner et al., 1987; Kabencell et al., 1990). Mammalian ARF binds GTP but does not contain a detectable GTPase activity (Weiss et al., 1989). The apparent guanine nucleotide off-rates for Sar1p are similar for GDP and GTP (0.07 min⁻¹ and 0.09 min⁻¹, respectively) and slower for GTPyS (0.04 min⁻¹). Nucleotide off-rates for different GTPases vary and for a given GTPase may be quite different for GTP and GDP. For example, Sec4p shows an off-rate for GDP (0.23 min⁻¹) that is much faster than GTP (0.002 min⁻¹) (Kabencell et al., 1990). N-ras exhibits off-rates for GTP and GDP of 0.006 min⁻¹ and 0.025 min⁻¹, respectively (Neal et al., 1988).

Although it is tempting to speculate how these altered rates of binding and hydrolysis translate into cellular function, increasing evidence suggests these parameters are tightly regulated in vivo by guanine nucleotide regulatory proteins (Bourne et al., 1991). In the case of yeast Ras1p, a guanine nucleotide regulatory protein that activates GTPase activity is encoded by the IRA1/2 genes (Tanaka et al., 1990) while acceleration of GDP release is accomplished by the CDC25 gene product (Crecet et al., 1990; Jones et al., 1991). Another type of guanine nucleotide regulatory protein is reported to bind a small GTPase and slow the release of guanine nucleotide (Ueda et al., 1990). Thus, Sar1p hydrolysis and exchange rates are likely to be modulated in vivo dependent on context. A number of gene products involved in ER vesicle formation in yeast have been associated genetically with SARI and could modulate Sar1p guanine nucleotide interactions. Characterization of purified Sar1p binding and hydrolysis properties now allows us to investigate modulation by Sec proteins in vitro. We have recently found that Sec23p stimulates the GTP hydrolysis rate of Sar1p. Sec23p is also required for transport vesicle formation from the ER, suggesting GTP hydrolysis by Sar1p is required for this event.

Sar1p preloaded with GTPyS was less active in promoting vesicle formation than Sar1p-GTP. This observation is quite similar to that reported for GTPyS inhibition of a budding reaction with a wild-type cytosol (Rexach and Schekman, 1991). A cytosol that has been gel-filtered to remove both Sar1p and GTP does not support vesicle formation unless supplemented with GTP or GDP and Sar1p. GTPyS cannot replace GTP in this assay. We propose that GTP hydrolysis is required for vesicle budding; however, we cannot exclude the possibility that the Sar1p-GTPyS form malfunctions due to an inability to interact with the transport machinery.

As a majority of cellular Sar1p is membrane bound, it is interesting to note that soluble Sar1p is required for efficient vesicle formation in vitro. This suggests that Sar1p cycles between soluble or membrane-bound forms and vesicle budding is regulated by the attachment of soluble Sar1p to its target site on the ER membrane. Sec12p facilitates the attachment of Sar1p to membranes (d'Enfert et al., 1991b), perhaps by catalyzing the exchange of GDP to GTP on Sar1p. Membrane-bound Sar1p-GTP then could function in the assembly of a protein complex required for vesicle budding. After the complex has executed its function, Sar1p hydrolysis of the bound GTP may result in a disassembly of the complex and a recycling of budding components. The failure of endogenous membrane-bound Sar1p to function in the absence of the cytosolic Sar1p remains unexplained. Perhaps an additional rate-limiting recycling factor is necessary to mobilize membrane-bound Sar1p. The function of Sar1p in vesicle budding can be investigated more directly once the in vitro vesicle formation assay is reconstituted with purified components.

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