Reconstitution of GTP-binding Sarl Protein Function in ER to Golgi Transport

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Abstract. In the yeast secretory pathway, two genes SEC12 and SARI, which encode a 70-kD integral membrane protein and a 21-kD GTP-binding protein, respectively, cooperate in protein transport from the ER to the Golgi apparatus. In vivo, the elevation of the SARI dosage suppresses the temperature sensitivity of the sec12 mutant. In this paper, we show cell-free reconstitution of the ER-to-Golgi transport that depends on both of these gene products. First, the membranes from the sec12 mutant cells reproduce temperature sensitivity in the in vitro ER-to-Golgi transport reaction. Furthermore, the addition of the Sarl protein completely suppresses this temperature-sensitive defect of the sec12 membranes. The analysis of Sarl partially purified by E. coli expression suggests that GTP hydrolysis is essential for Sarl to execute its function.
The duplication of SARI suppresses not only sec12 but also another ER-Golgi mutant sec17. Kaiser and Schekman (1990) have reported that SEC12 and SEC16 show extensive genetic interaction with each other as well as with SEC13 and SEC23. Furthermore, mutations in these genes exhibit a similar morphological phenotype: no accumulation of small vesicles which may be an intermediate in the ER-to-Golgi transport. These observations might suggest that Sarlp executes its function with Sec12p and other gene products in the earliest event of the transport, the formation of vesicles from the ER membrane.

To better understand the roles of these gene products in vesicular transport, cell-free systems provide a powerful tool. Baker et al. (1988) and Ruohola et al. (1988) have developed yeast in vitro assays that measure transport of α-factor precursor from the ER to the Golgi apparatus, using gently lysed yeast spheroplasts (semi-intact cells). In vivo studies have established that the mating pheromone α-factor is subject to sequential modification and processing during transit through the secretory pathway (Fuller et al., 1988). The ER-to-Golgi in vitro assays monitor the addition of outer chain carbohydrate which is the indication of the arrival of α-factor precursor in the Golgi apparatus. Using these assays, two components Sec23p and Ypt3p, have been proved to be involved in this transport step (Baker et al., 1988; Ruohola et al., 1988; Bacon et al., 1989; Baker et al., 1989).

In this article, we report that the membranes prepared from the sec12 mutant show temperature sensitivity in vitro. This temperature-sensitive reaction can be remedied by the addition of Sarlp in a dose-dependent manner, which is the reproduction of the in vivo phenomenon executed by Sarlp. Furthermore, this effect of Sarlp is abolished when the non-hydrolyzable GTP analogue, guanosine 5'-O-(3-thiotriphosphate) (GTPγS), is prebound to the protein.

Materials and Methods

Materials

The yeast strains used in this study were X2180-1A (mal2-1 CUP1 MATa) (Yeast Genetic Stock Center, Berkeley, CA), MBY10-7A (sec12-4 ura3-52 leu2-3, 112 trpl-289 his3 his4 ura2 MATa) (Nakanoo et al., 1988), and ANY26 (sar1:URA4 ura3-52 leu2-3, 112 trpl-289 his3 his4 MATa[pGALL-SARI TRP1]) (Nakano and Muramatsu, 1989). An Escherichia coli B strain, BL21 (DE3) [F-ompT raf (DE3)] carrying pLysS (Studier et al., 1990) was used for expression of the SARI gene product.

Yeast cells were grown in YP medium [1% Bacto-Yeast Extract (Difco Laboratories, Inc., Detroit, MI) and 2% Polypeptone (Nihon Pharmaceutical Co. Ltd., Tokyo, Japan)] containing 2% glucose (YPD) or 5% galactose and 0.2% sucrose (YPGS). SEC strains were cultured at 30°C, sec2 strain at 24°C. E. coli cells were grown in LB medium (0.5% Bacto-Yeast Extract, 1% Polypeptone, and 1% NaCl) at 37°C.

Anti-α-factor mannose antisera was prepared as described (Bailou, 1970). Anti-α-factor antisera was prepared using the β-gal-α-factor fusion protein as an antigen (Rothblatt and Meyer, 1986).

In Vitro Transcription and Translation of α-Factor Precursor

The plasmid pDI100 containing the MFod gene under the SP6 promoter was subjected to in vitro transcription as described (Hansen et al., 1986). Reaction was carried out in 40 mM Tris-HCl (pH 7.4), 6 mM MgCl2, 2 mM spermidine, 10 mM DTT, 0.1 mg/ml BSA, 500 U/ml RNase inhibitor, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.04 mM GTP, 0.25 mM 5'GpppG [5'-S-(3'-methyl-guanosine)-5'-S-guanosine triphosphate], 630 U/ml SP6 RNA polymerase, and 0.08 mg/ml template DNA.

Translation-competent yeast lysate was prepared from X2180-1A as described (Deshaies and Schekman, 1989). In vitro translation was performed at 20°C for 50 min, basically following the protocol of Baker et al. (1988). A 2.5-ml reaction contained >250 µg prepro-α-factor mRNA, 1 ml yeast lysate, 37 Mbq 35S methionine, 660 U RNase inhibitor, 20 mM Hepes-KOH (pH 7.4), 115 mM KCl, 35 mM NH4OAc, 3 mM Mg(OAc)2, 1 mM ATP, 0.1 mM GTP, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 3 mM DTT, 30 µM each of the 19 amino acids excluding methionine, 0.2 mg/ml yeast tRNA, and 5.6% glycerol.

In Vitro Transport Reaction

Transport-competent semi-intact cells and cytosol were prepared exactly as described (Baker et al., 1988). Transport reaction in vitro was also performed according to the method of Baker et al. (1988) with some modifications. In the standard assay condition, a 50-µl reaction contained 130 µg of cytosol, 70 µg of labeled prepro-α-factor (>300,000 cpm), 9 × 107 semi-intact cells, 50 µM GDP-mannose, 1 mM ATP, 40 mM creatine phosphate, and 0.2 mg/ml creatine phosphokinase in the reaction buffer (20 mM Hepes-KOH [pH 6.8], 150 mM KCl, 250 mM sorbitol, and 5 mM Mg(OAc)2). Immunoprecipitation with anti-α-factor or anti-α-1-6 mannose antibody was performed as described (Baker et al., 1988). The immunoprecipitates were subjected to either SDS-PAGE and fluorography or scintillation counting using solid scintillator Ready Cap (Beckman Instruments Inc., Palo Alto, CA). To prepare cytosol containing a large amount of Sarlp, ANY26 cells which have SARI expression under control of the GAL1 promoter were cultured in YPO9 and lysed.

Expression of Wild-type and Mutant Sarlp in E. coli

To make Sarlp produced in E. coli, the cDNA clone of SARI (pAN3Y3-5; see Nakano and Muramatsu, 1989) was engineered. First, an Ncol site (CCATGG) was added to the initiator ATG codon of SARI by changing TA (-2 to -4) numbers refer to the nucleotide positions in the genomic sequence starting from the translation initiation site) to CC using Amersham's site-directed mutagenesis kit (Amersham Corp., Arlington Heights, IL). From this plasmid (pAN3Y3-13), the 0.6-kb Ncol-EcoRI fragment containing the whole coding sequence of SARI and ~400-bp 5'-flanking sequence was excised out and inserted into the Ncol/BamHI sites (EcoRI and BamHI ends blunted) of pET3-d (Studier et al., 1990) to give the wild-type Sarlp expression plasmid, pAN3Y3-14. To make the mutant version of Sarlp, the nucleotides GAT (356-358) were converted to GTT in pAN3Y3-5 by site-directed mutagenesis, which will yield the Asp-73 to Val point mutation (Sarlp<sup>Asp73Val</sup>). The 0.6-kb Ncol-EcoRI fragment with the mutation was transferred to the expression vector pMY2 which was derived from pET3-d by disrupting the preexisting EcoRI site in the vector by Klenow filling-in and reintroducing a new EcoRI site by linker ligation at the BamHI site. The resulting plasmid was named pMY3E3-3. To confirm the plasmid construction, the whole region from promoter to terminator was sequenced.

The expression plasmids were then introduced into the E. coli strain BL21 (DE3) which had been transformed with another plasmid pLysS (Studier et al., 1990). The cells containing both pLysS and the SARI expression plasmid (pAN5Y3-14 or pMY3E3-3) were selected and cultured at 37°C in LB medium containing 50 µg/ml ampicillin and 25 µg/ml chloramphenicol. In the early log phase, isopropyl-1-thio-β-D-galactoside (IPTG) was added at the final concentration of 1 mM to invoke induction of SARI and the incubation continued for 60 min. Cells were harvested, lysed by addition of 2% SDS and brief bath sonication, and subjected to SDS-PAGE.

The GTP-blotting experiments were performed as described (Lapetina and Reep, 1987). Typically, the binding reaction buffer contained 0.66 mM [α-32P]GTP (74 kBq/ml) in 30 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 1 mM EGTA, and 0.3% Tween20.

Partial Purification of Sarlp from E. coli Lysate

When the expression of SARI was fully induced in E. coli, the product protein showed a tendency to form aggregates. Sarlp was partially purified from the aggregates according to the method of Matsuyama et al. (1990) with some modifications. The E. coli cells harboring pLysS and either of pAN5Y3-14 and pMY3E3-3 were grown in 250 ml LB medium supplement

1. Abbreviation used in this paper: IPTG, isopropyl-1-thio-β-D-galactoside.
with 50 μg/ml ampicillin at 37°C and induced for Sarlp synthesis at the early log phase by incubation with 1 mM IPTG for 60 min. Cells were harvested, suspended in 50 mM Tris-HCl (pH 7.4)/0.5 mM PMSF, frozen at −80°C for 30 min, and thawed quickly in a 30°C water bath. The suspension was subjected to vigorous sonication (Branson Sonifier Model B-12; Branson Sonic Power, Danbury, CT) (5 × 1-min pulses on ice at 100 W) and centrifuged at 12,000 g for 10 min. Pellet was suspended in 50 mM Tris-HCl (pH 7.4), spun again at 12,000 g for 10 min, and resuspended in 150 μl of 6 M guanidine-HCl/50 mM Tris-HCl (pH 7.4). A 50-μl aliquot of the clear supernatant was diluted 10-fold with the transport reaction buffer containing 1 mM GDP (GTP or GTPγS) and 1 mM DTT, and dialyzed against five changes (50 ml each) of the reaction buffer containing 0.1 mM GDP (GTP or GTPγS) and 0.1 mM DTT. In the case of GTPγS, the concentration in the last dialysis buffer was lowered to 0.01 mM. The dialyzed samples were directly used for transport assays. About 1 mg of the Sarlp fraction was obtained from a 250-ml culture.

Results

Semi-intact Cells of sec12 Have a Temperature-Sensitive Defect in ER-to-Golgi Transport

The sec12 temperature-sensitive mutant cells do not transport proteins from the ER to the Golgi apparatus at restrictive temperature (Novick et al., 1980). To manifest the defect of the sec12 mutant, its transport activity was tested in vitro.

Reconstitution of the ER-to-Golgi transport reaction was performed according to the method of Baker et al. (1988). The wild-type and sec12 semi-intact cells were prepared from X2180-1A (sec12-) and MBY10-7A (sec12Δp) cells which were grown at 30 and 24°C, respectively. Cytosol was prepared from the wild-type cells, because Sec12p is known to be an integral membrane protein (Nakano et al., 1988). Either the wild-type or sec12 semi-intact cells were mixed with cytosol, 35S-labeled prepro-α-factor and ATP in the reaction buffer, and incubated at 20°C or 26°C for 60 min. As shown in Fig. 1, the conversion of prepro-α-factor to the ER and Golgi forms occurred in the wild-type semi-intact cells both at 20 and 26°C (lanes 1 and 3). The antibody specific to α1-6 mannose linkage (α1-6 Man), which constitutes a major epitope of the outer-chain carbohydrate, immunoprecipitated the Golgi form only (Fig. 1, lanes 2 and 4). In the sec12 semi-intact cells, the processing to the ER and Golgi forms normally took place at 20°C (Fig. 1, lanes 5 and 6). At 26°C, however, the Golgi form was not detected at all while the ER form was produced (Fig. 1, lanes 7 and 8). This indicates that in the sec12 semi-intact cells α-factor precursor was translocated across the ER membrane but not transported to the Golgi at 26°C. As shown below (Fig. 3), the sec12 semi-intact cells exhibited marked defect in the Golgi-species formation over a wide range of temperatures and little activity was detected above 26°C. This temperature sensitivity was linked to the sec12Δp mutation; the semi-intact cells from the wild-type strain ANY21 which is completely isogenic with MBY10-7A except the SEC12 locus (Nakano and Muramatsu, 1989) showed the wild-type phenotype (data not shown). Although the restrictive condition was somewhat shifted to lower temperatures than in vivo (see below), we concluded that the temperature sensitivity of sec12 was reproduced in vitro.

Suppression of the Temperature Sensitivity of sec12 by Cytosol from Sarlp Overproducer

With the temperature-sensitive reaction of the sec12 semi-intact cells in hand, we proceeded to the analysis of Sarlp, the suppressor of sec12. We have recently shown that Sarlp is tightly associated with membranes and only a small pool is detected in the wild-type cytosol (Nishikawa and Nakano, 1991). However, when the SARI gene is overex-

Figure 1. sec12 semi-intact cells show temperature-sensitive ER-to-Golgi transport in vitro which can be suppressed by cytosol containing excess Sarlp. The wild-type (WT) or sec12 semi-intact cells (Membrane), 35S-labeled prepro-α-factor, and the wild-type cytosol were incubated in the reaction buffer at 20 or 26°C for 60 min (see Materials and Methods for details). Cytosol (30 μg) from the cells overproducing Sarlp (SarlpΔp) was also added to the reaction mixture at the beginning of the incubation (lanes 9 and 10). The total amount of cytosol was kept to 200 μg protein in total. After 60 min, reaction was stopped by addition of SDS and the mixture was subjected to immunoprecipitation with α-factor (αF) or anti-α1-6 mannose (α1-6 Man) antibody. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. Prepro-α-factor migrates at 20 kD and the 26-kD band corresponds to the ER form of pro-α-factor. The smear species ranging from 30 to ~100 kD is the Golgi form that can be precipitated with anti-α1-6 Man antibody. A light shadow around 46 kD is due to the heavy chain of antibodies.

Figure 2. Dose dependence on cytosol from Sarlp overproducer of the suppression of sec12. The sec12 semi-intact cells, [35S]prepro-α-factor and wild-type cytosol were incubated with the indicated amount of cytosol from the Sarlp overproducer at 26°C for 60 min. The Golgi form produced was analyzed by immunoprecipitation with anti-α1-6 Man antibody and scintillation counting. The total amount of cytosol was adjusted to 200 μg in all reactions. The radioactivity of the immunoprecipitates assayed at 20°C with the wild-type cytosol alone was 1,575 ± 255 cpm. Bars indicate deviations in a duplicate experiment.
pressed by the GAL1 promoter, the cytosolic level of Sarlp is highly elevated. Using this cytosol from the Sarlp overproducer (hereafter referred to as Sarlp-cytosol), the effect on the temperature sensitivity of sec12 semi-intact cells was tested. As shown in Fig. 1, lanes 9 and 10, the addition of Sarlp-cytosol remarkably alleviated the defect of the Golgi-form production in the sec12 semi-intact cells at 26°C. The Golgi-form formation was dependent on the amount of the added Sarlp-cytosol (Fig. 2). The ability to restore the reaction increased almost linearly in the range shown in the figure. About 25 μg cytosol per 50 μl reaction mixture was enough to achieve the activity level of 20°C. Thus, the temperature sensitivity of sec12 in vitro was perfectly suppressed by the cytosol containing excess amounts of Sarlp.

Temperature Dependence of Transport

The in vitro suppression of sec12<sup>−</sup> was further analyzed by examining the temperature dependence profiles of the wild-type and sec12 semi-intact cells. As shown in Fig. 3 A, the ER-to-Golgi transport activity varied with temperature even in the wild type. At temperatures higher than 24°C, the activity decreased gradually (Fig. 3 A, solid circles). In the sec12 semi-intact cells, however, the transport activity drastically dropped from 20 to 26°C (Fig. 3 B, open circles), highlighting the temperature sensitivity of sec12. This difference in the temperature profile is not due to the Sarlp level; the amount of Sarlp was almost the same in the wild-type and sec12 semi-intact cells as estimated by immunoblotting (not shown).

When the Sarlp-cytosol was added to the sec12 semi-intact cells, remarkable restoration of the reaction was observed over the wide range of temperatures (Fig. 3 B, open triangles). Only a slight increase by Sarlp-cytosol was observed for the wild-type semi-intact cells at all temperatures (Fig. 3 A, solid triangles). With the Sarlp-cytosol, the temperature-dependence profile of the sec12 semi-intact cells is almost superimposable on the wild-type profile. At 20°C, the activity of the sec12 semi-intact cells was still significantly improved by Sarlp-cytosol, suggesting that the sec12 mem-

branes are partially defective even at this low temperature. Such suppression effect was not observed below 16°C (not shown).

Expression of Sarlp in E. coli as a GTP-binding Protein

To unambiguously demonstrate that it is Sarlp that suppresses sec<sub>12</sub><sup>−</sup>, we decided to make Sarlp in E. coli. The wild-type cDNA clone of SARI (Nakano and Muramatsu, 1989) was subcloned into the pET3-d expression vector (Studier et al., 1990) and the resultant plasmid pANY3-14 was introduced into the E. coli strain BL21 (DE3)/pLysS. In the transformant cells, expression of SARI is regulated by the T7 promoter while the T7 polymerase is under the lac promoter control, so that the synthesis of Sarlp should be induced by IPTG. The E. coli cells harboring either the expression plasmid pANY3-14 or the vector pET3-d were incubated with or without IPTG, and lysates were prepared and subjected to SDS-PAGE. As seen in Fig. 4 A, a marked production of a 23-kD protein was observed by IPTG induction of the SARI-expression plasmid (Fig. 4 A, lane 4, arrowhead). This species is in fact Sarlp as proved by immunoblotting with the anti-Sarlp antibody (Fig. 4 B, lane 8). A small amount of Sarlp was synthesized in the absence of IPTG due to the leak of lac repression (Fig. 4 B, lane 7).

In a previous paper, we reported that SARI encodes a GTP-binding protein based on the striking conservation of GTP-binding sequence motifs (Nakano and Muramatsu, 1989). To give a biochemical proof that Sarlp indeed binds GTP, a GTP-blotted experiment was conducted for these E. coli lysates (Fig. 4 C). The lysates of the IPTG-induced cells harboring the SARI-expression plasmid (Fig. 4 C, E) or the vector alone (V) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane as in the immunoblotting. The nitrocellulose was incubated with α-<sup>32</sup>P-labeled GTP (Fig. 4 C, lanes 9 and 10), [α-<sup>32</sup>P]-ATP (lanes 11 and 12), or [α-<sup>32</sup>P]-GTP in the presence of 30-fold excess cold ATP (lanes 13 and 14), washed, and autoradiographed. The result clearly indicates that Sarlp does bind GTP and the binding is specific to GTP; ATP could not be bound to Sarlp nor did it effectively compete with GTP binding. Thus Sarlp was efficiently produced in E. coli with the capability of GTP-specific binding.

Partial Purification of Wild-type and Mutant Sarlp Made in E. coli

The E. coli lysate containing Sarlp was tested for the ability to suppress sec<sub>12</sub><sup>−</sup> in the in vitro ER-to-Golgi transport reaction described above. However, E. coli lysates were found to contain potent inhibitor(s) to this yeast in vitro reaction. So we attempted to purify Sarlp from the E. coli lysate, taking advantage of the fact that it tends to form aggregates when overproduced. E. coli cell lysate was prepared by freeze-thawing and sonication and centrifuged at 12,000 g. About 60% of Sarlp was recovered in the pellet. The pellet was solubilized with 6 M guanidine-HCl and cleared of insoluble materials by ultracentrifugation. The supernatant was diluted 10-fold with the transport reaction buffer containing 1 mM GDP and dialyzed against the buffer containing 0.1 mM GDP. The obtained Sarlp fraction as well as the initial crude lysate were subjected to SDS-PAGE and Coomassie blue staining (Fig. 5 A, lanes 1 and 2). From densito-

Figure 3. Temperature-dependence profiles of wild-type and sec12 semi-intact cells with or without Sarlp-cytosol. The wild-type (A) or sec12 (B) semi-intact cells, [35S]prepro-α-facotor, and wild-type cytosol were mixed with (A, Δ) or without (●, ○) 60 μg cytosol from the Sarlp overproducer and incubated for 60 min at the indicated temperatures. The total amount of cytosol was kept to 200 μg constant. The Golgi form produced was analyzed by immunoprecipitation with anti-α-Man antibody and scintillation counting.
Figure 4. Expression of Sarlp in E. coli and GTP-binding analysis. The E. coli cells harboring either the SARI expression plasmid (pANY3-14) or the vector alone (pET3-d) were incubated with or without 1 mM IPTG for 60 min. The cells were lysed with 2% SDS and subjected to SDS-PAGE. Gels were stained with Coomassie blue (A), analyzed by immunoblotting with the anti-Sarlp antibody (B), or subjected to nucleotide blotting (C). In the nucleotide blotting experiment, lysates from IPTG-induced cells harboring pANY3-14 (E) or pET3-d (V) were electrophoresed under a nonreducing condition, transferred to a nitrocellulose membrane and incubated with 0.66 nM [α-32P]GTP (GTP*), 0.66 nM [α-32P]ATP (ATP*), or 0.66 nM [α-32P]GTP plus 20 nM cold ATP (GTP*/ATP). Arrowheads indicate Sarlp.

We have been trying to construct a variety of sarl mutants by site-directed mutagenesis. Several mutants with a single amino acid replacement in the GTP-binding consensus sequences were made and subjected to the above partial purification of Sarlp (Yamagishi, M., S. Nishikawa, T. Oka, and A. Nakano, unpublished data). Among them, the mutant D73V (replacement of Asp-73 by Val) showed a similar profile to the wild type in expression and purification from the E. coli lysate (Fig. 5A, lanes 3 and 4). The Asp-73 residue of Sarlp corresponds to Asp-57 of mammalian ras proteins and is perfectly conserved over the whole GTP-binding protein superfamily. Fig. 5B shows the GTP blotting analysis of the wild-type and the D73V mutant E. coli lysates. The replacement of the Asp-73 residue by valine completely abolished the GTP-binding activity of Sarlp under this condition (Fig. 5B, lane 6). The partially purified wild-type Sarlp (lane 2) retained the normal GTP-binding activity (not shown).

Sarlp Itself Is Essential for the Suppression of sec12

The Sarlp fractions partially purified from E. coli lysates were assayed for the activity of suppressing sec12. Fig. 6 shows the formation of the Golgi form of pro-α-factor at 26°C in the in vitro transport reaction. The wild-type Sarlp fraction prepared from E. coli lysate did suppress the temperature sensitivity of the sec12 semi-intact cells (Fig. 6, solid circles). The suppression was dose dependent and the reaction reached the level of 20°C at 1.1 μg protein. In contrast, the mutant SarlpD73V did not show the suppression activity at all (Fig. 6, solid triangles). Fig. 7A shows that the E. coli wild-type Sarlp fraction in fact gave rise to the smearable Golgi form of pro-α-factor whereas the SarlpD73V fraction did not (Fig. 7A, compare lanes 3 and 4). The 26-kD ER form was normally produced in the presence of SarlpD73V (Fig. 7A, lane 4). Since the sole difference between the two fractions was due to the single mutation Asp-73 to Val in Sarlp, these observations give definitive evidence that it is Sarlp that functions in the suppression of sec12 in vitro.

GTP Hydrolysis Is Required for Sarlp Function

In accordance with the fact that Sarlp is a GTP-binding protein, either GTP or GDP was required in the renaturation process of the guanidine-solubilized E. coli Sarlp. Sarlp that was solubilized and dialyzed without GTP or GDP did not show the activity to suppress sec12 (not shown). It is pos-
sible that the mutant SarlpD73V is functionless because it cannot attain correct folding during the renaturation process due to the defect of GTP (or GDP) binding. The wild-type Sarlp showed almost the same activity in sec12 suppression regardless of whether GDP or GTP was prebound to it (see Table I). It remains to be determined whether both Sarlp-GDP and Sarlp-GTP are active in the in vitro reaction or Sarlp-GDP has to be converted to the GTP form during the reaction.

To test whether the hydrolysis of GTP is required for the function of Sarlp, we examined the effect of GTPyS, the non-hydrolyzable analogue of GTP. The wild-type Sarlp was renatured in the presence of GTPyS and dialyzed against the buffer containing GTPyS (Sarlp-GTPyS). As shown in Table I, the temperature sensitivity of the sec12 semi-intact cells was not suppressed by this Sarlp-GTPyS (see also Fig. 7 B, lane 7). In the experiments of Table I and Fig. 7 B all reactions contained free GTPyS at 0.94 and 1.1 μM respectively. Although GTPyS inhibits the in vitro ER-to-Golgi transport reaction at high concentrations (data not shown; see also Baker et al., 1988; Ruohola et al., 1988), this low level of GTPyS showed only a marginal effect on the ER-to-Golgi transport. Even in the presence of 1.1 μM GTPyS, the cytosol from the Sarlp-overproducing yeast completely suppressed sec12 (Fig. 7 B, lane 8), indicating that the deficiency of suppression was due to the defect of Sarlp-GTPyS itself. These results suggest that the hydrolysis of GTP is required for the action of Sarlp in sec12 suppression.

Discussion

In this report, we have taken advantage of the yeast ER-to-Golgi in vitro transport assay to demonstrate that semi-intact cells from sec12 are temperature sensitive in this transport reaction and that this defect is perfectly cured by the elevated level of Sarlp. Since SARI has been identified by its ability to suppress the temperature sensitivity of sec12 in vivo, this in vitro phenomenon is regarded as a reproduction of the physiological function of Sarlp. At the restrictive temperature of the sec12 membranes, the transport of α-factor precursor from the ER to the Golgi apparatus is totally dependent on the amount of Sarlp.

Interaction between Sec12p and Sarlp

It is known that the genes SECl2 and SARI encode a 70-kD integral membrane protein and a 21-kD GTP-binding protein, respectively, both of which reside mainly in the ER, and that they are interacting with each other probably at the prod-
Figure 7. Evidence for the GTP-dependent suppression of sec12
to Sarlp by Sarlp. The sec12 semi-intact cells, [35S]prepro-α-factor and the wild-type yeast cytosol were incubated at 20 or 26°C for 60 min. Some reactions contained 1.1 μg of the E. coli fractions: SarlpWT

Table I. Sarlp-GTPyS Cannot Suppress sec12

| Temperature | Sarlp | GDP* | GTP* | GTPyS* |
|-------------|-------|------|------|--------|
| 20°C        | -     | 2,781 ± 133 | 2,953 ± 135 | 2,753 ± 57 |
| 26°C        | -     | 751 ± 147  | 654 ± 22   | 820 ± 50  |
| 26°C        | +     | 2,413 ± 321 | 2,782 ± 228 | 1,170 ± 45 |

The numbers are shown with standard deviations in duplicate experiments. When the reaction was depleted of ATP as a control, the radioactivity precipitated was 228 ± 74 cpm. * The same procedure was repeated as above except that 1 mM and 0.01 mM GDP or GTP was added to other reactions to prevent metabolism and slow reassociation, and the dialyzed buffer (+) was added to the reaction, which gave the final concentration of free GDP or GTP at 1 μM.

E. coli Sarlp was renatured in the presence of 1 mM GDP (or GTP) and dialyzed against 0.1 mM GDP (or GTP). The dialyzing buffer (−) or the dialyzed Sarlp-GDP (or Sarlp-GTP) (+) was added to the reaction, which gave the final concentration of free GDP (or GTP) at 1 μM. The same procedure was repeated as above except that 1 mM and 0.01 mM GDP or GTP was present during renaturation and the last dialysis, respectively, and the final concentration of free GTPyS in the reaction was 0.94 μM.
cause it fails to renature due to the defect of GTP-binding. Nevertheless, the fact that the single mutation abolished the ability of Sarlp to suppress sec12 is unambiguously indicated that it is Sarlp itself but not a contaminating component that promotes transport from the ER to the Golgi apparatus in the in vitro reaction.

**Role of GTP Hydrolysis**

The wild-type Sarl protein produced in *E. coli* has been further used to test the role of GTP hydrolysis in its function. Sarl that is renatured in the presence of GTPγS instead of GTP or GDP shows only a little activity to suppress sec12 in the assay. This inhibition is not due to free GTPγS that is present in the assay, because the addition of the active form of Sarlp gives the full recovery of the transport. Sarlp complexed with GTPγS is responsible for the defect. Since GTPγS is the analogue of GTP which is hydrolyzed extremely slowly, this observation suggests that GTP hydrolysis is essential for Sarlp function. We are aware that we cannot exclude the possibility that Sarlp-GTPγS is inactive because it is not renatured well. Further analysis requires knowledge of kinetic parameters such as the affinity and exchange rate for each guanine nucleotide and, for this purpose, complete purification of Sarlp is currently under way.

**Modification of Sarlp**

Many small GTP-binding proteins are modified posttranslationally. As a typical example, ras proteins have the -CAAX motif at the COOH terminus which receives the farnesylation by the action of farnesyl transferase (probably encoded by DFR1/RAM1 and RAM2 in yeast) (Schäfer et al., 1990; Goodman et al., 1990). Yptl or Rab family proteins have the -CC or -CXC motif at the COOH terminus in common and this sequence is believed to play important roles for membrane attachment by palmitoylation or other modifications (Molenaar et al., 1988; Walworth et al., 1989). Furthermore, some α-subunits of G proteins and ARF proteins conserve an NH2-terminal consensus sequence for myristoylation (Buss et al., 1987; Sewell and Kahn, 1988). Sarlp does not contain any of these sequences (Nakano and Muramatsu, 1989). Nevertheless, Sarlp is very tightly associated with membranes; 0.1 M sodium carbonate or 1 M NaCl is not able to solubilize Sarlp, 1% Triton X-100 or 2 M urea can only partially solubilize Sarlp, and only 1% deoxycholate can thoroughly release Sarlp from membranes (Nishikawa and Nakano, 1991). This suggests a novel mechanism of membrane attachment of Sarlp. The fact that Sarlp produced in *E. coli* is sufficiently active in the promotion of ER-to-Golgi transport in vitro may imply that posttranslational modification is not required for the Sarlp function. Of course, we cannot rule out the possibility that Sarlp is modified during the assay.

**Subreactions in the ER-to-Golgi Transport**

As Baker et al. (1988) have already noticed, the ER-to-Golgi transport reaction by semi-intact cells shows an inherent temperature-dependent profile even for the wild type. For some unknown reasons, the wild-type membranes are less active at 30 than at 20°C, though 30°C is the optimal condition for the wild-type cell growth. In contrast, thermosensitivity of the sec12 semi-intact cells is quite distinctive. As clearly seen in Fig. 3, the sec12 membranes show a severe defect at lower temperatures. In the reactions of sec12 membranes defective in the formation of the Golgi-form pro-α-factor, the ER form is normally produced indicating that the defect of the sec12 membranes is specific to the ER-to-Golgi transport. Then, in which subreaction of the transport are the sec12 membranes impaired?

The vesicular transport from the ER to the Golgi apparatus is thought to consist of many substeps: budding and fission of transport vesicles from the ER membrane; transport and targeting of the vesicles to the Golgi; and attachment and fusion of the vesicles with the Golgi membrane. Kaiser and Schekman (1990) have argued according to their morphological observations that sec12 is one of the mutants that are defective in vesicle formation. No accumulation of small vesicles was seen in the sec12 cells at the restrictive temperature. Recently, in vitro assays to assess formation of vesicles from the ER membrane have been devised by measuring movement of α-factor precursor from semi-intact cells to the fraction that needs higher speed for sedimentation (Groesch et al., 1990; Rexach and Schekman, 1991). Rexach and Schekman (1991) have also shown that the sec12 membranes are temperature sensitive for the movement of pro-α-factor from the semi-intact cells to the slowly sedimenting fraction, suggesting that Sec12p is in fact involved in the formation of intermediate vesicles. Since the increase of Sarlp completely suppresses the defect of sec12 membranes and enables the transport all the way to the Golgi apparatus, it may be reasonable to assume that Sarlp exerts its function at the same step as Sec12p, perhaps the vesicle formation. This idea is consistent with our observation that Sarlp is mainly located on the ER membrane (Nishikawa and Nakano, 1991). However, it is still an open question whether these gene products are required only for the earliest reaction or involved in multiple steps. In this regard, it is intriguing that immunofluorescence of Sarlp stains some dot-like structures in addition to the ER, suggesting its multiple localizations in the cell (Nishikawa and Nakano, 1991). Obviously, we need further dissection of the ER-to-Golgi transport reaction to address this problem. We are currently trying to determine which particular subreaction requires GTP hydrolysis by Sarlp.

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