Reconstitution of GTP-binding Sar1 Protein Function in ER to Golgi Transport

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Abstract. In the yeast secretory pathway, two genes *SEC12* and *SAR1*, 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 *SAR1* dosage suppresses 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 Sar1 protein completely suppresses this temperature-sensitive defect of the *sec12* membranes. The analysis of Sar1p partially purified by *E. coli* expression suggests that GTP hydrolysis is essential for Sar1p to execute its function.

GUANOSINE triphosphate-binding proteins or GTPases constitute an expanding superfamily, each member of which acts as a molecular switch in a wide variety of cellular functions (Bourne et al., 1990, 1991). Starting from the work on polypeptide chain elongation factors, extensive studies have established the role of GTP hydrolysis in many regulatory reactions. Very recently, a new frontier has emerged from the discovery of a number of GTP-binding proteins involved in the protein secretory pathway.

In yeast *Saccharomyces cerevisiae*, genetic approaches have identified dozens of genes that function in the intracellular routes of protein traffic. To our knowledge, at least seven genes encode GTP-binding proteins that are involved in the secretory pathway. *SRH1* (*SRP54*) codes for the yeast counterpart of the 54-kD subunit of the signal recognition particle (Amaya et al., 1990; Hann et al., 1989) and may be important for protein translocation across the membrane of the endoplasmic reticulum. *SAR1*, whose product (Sar1p, 21 kD) resides mainly on the surface of the ER membrane, is required for protein transport from the ER to the Golgi apparatus, and its elevated gene dosage suppresses mutations occurred in other secretory components (Nakano and Muramatsu, 1989; Nishikawa and Nakano, 1991). *YPT1*, *ARF1*, and *ARF2* encode a set of small GTP-binding proteins that are also implicated in the ER-to-Golgi transport, although their localization is suggested to be rather in the Golgi apparatus (Segev et al., 1988; Schmitt et al., 1988; Stearns et al., 1990). The *SEC4* gene product is required for the last event of secretion, the fusion of secretory vesicles with the plasma membrane (Salminen and Novick, 1987). *VPS1*, which plays a role in sorting of vacuolar proteins from the main secretory pathway, codes for a homologue of dynamin, the GTP-driven motor associated with microtubules (Rothman et al., 1990; Obar et al., 1990). Roles of these GTP-binding proteins have been discussed from the viewpoints of accuracy of molecular recognition and unidirectionality of reactions. However, the significance of GTP hydrolysis by these proteins have not been directly addressed.

We have focused on the function of Sar1p, the GTP-binding protein that is required in the earliest step of vesicular transport. *SAR1* was originally isolated as the multicopy suppressor of *sec12*, a temperature-sensitive mutant defective in the ER-to-Golgi transport (Nakano et al., 1988; Nakano and Muramatsu, 1989). Using a conditional lethal mutant, *SAR1* has been shown to be required for exit of secretory and vacuolar proteins from the ER (Nakano and Muramatsu, 1989). The elevation of the *SAR1* gene dosage can suppress the temperature-sensitive defect of *sec12* but not the lethality of the *sec12* null mutation, suggesting the direct interaction of the two gene products. Although the physical interaction between Sec12p and Sar1p has yet to be demonstrated, several pieces of evidence support the view that they cooperate in the ER-to-Golgi transport by forming a complex. Sar1p is apparently cofractionated by differential centrifugation with Sec12p, which is a 70-kD integral membrane protein mostly residing in the ER (Nishikawa and Nakano, 1991). At least a large fraction if not all of Sar1p is located on the ER membrane as seen by immunofluorescence (Nishikawa and Nakano, 1991). Sar1p does not have either a stretch of hydrophobic amino acids typical for membrane association or sites for any known type of lipidic modifications, but is tightly associated with membranes perhaps by protein–protein interaction (Nishikawa and Nakano, 1991). The point mutation that confers the temperature-sensitive phenotype of *sec12* is located in the cytoplasmic domain of Sec12p (d'Enfert, C., C. Barlowe, S. Nishikawa, A. Nakano, and R. Schekman, manuscript submitted for publication), while overproduction of Sar1p increases the cytoplasmic pool of Sar1p (Nishikawa and Nakano, 1991). Furthermore, the increase of Sec12p seems to reduce the level of soluble Sar1p (Nishikawa and

Nakano, 1991; d'Enfert, C., C. Barlowe, S. Nishikawa, A. Nakano, and R. Schekman, manuscript submitted for publication).

The duplication of *SAR1* suppresses not only *sec12*^{ts} but also another ER-Golgi mutant *sec16*^{ts}. 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 Sar1p 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 Yptlp, 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 Sar1p in a dose-dependent manner, which is the reproduction of the in vivo phenomenon executed by Sar1p. Furthermore, this effect of Sar1p 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 (*mal gal2 CUP1 MATa*) (Yeast Genetic Stock Center, Berkeley, CA), MBY10-7A (*sec12-4 ura3-52 leu2-3, 112 trp1-289 his3 his4 suc gal2 MATa*) (Nakano et al., 1988), and ANY26 (*sar1::URA3 ura3-52 leu2-3, 112 trp1-289 his3 his4 MATα* [p*GAL1-SAR1 TRP1*]) (Nakano and Muramatsu, 1989). An *Escherichia coli* B strain, BL21 (DE3) [F^- *ompT* r_B^- m_B^- (DE3)] carrying pLysS (Studier et al., 1990) was used for expression of the *SAR1* 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, *sec12* 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-α1→6 mannose antiserum was prepared as described (Ballou, 1970). Anti-α-factor antiserum 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 pDJ100 containing the *MFα1* 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 $MgCl_2$, 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 m^7GpppG [P^1-5′-(7-methyl)-guanosine-P^3-5′-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 L-[^{35}S]methionine, 660 U RNase inhibitor, 20 mM Hepes-KOH (pH 7.4), 115 mM KOAc, 35 mM NH_4OAc, 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), 9×10^7 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 KOAc, 250 mM sorbitol, and 5 mM $Mg[OAc]_2$). Immunoprecipitation with anti-α-factor or anti-α1→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 Sar1p, ANY26 cells which have *SAR1* expression under control of the *GAL1* promoter were cultured in YPGS and lysed.

Expression of Wild-type and Mutant Sar1p in E. coli

To make Sar1p produced in *E. coli*, the cDNA clone of *SAR1* (pANY3-5; see Nakano and Muramatsu, 1989) was engineered. First, an NcoI site (CCATGG) was introduced at the initiator ATG codon of *SAR1* by changing TA (-2 to -1: 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 (pANY3-13), the 0.6-kb NcoI-EcoRI fragment containing the whole coding sequence of *SAR1* and ~40-bp 3′-flanking sequence was excised out and inserted into the NcoI/BamHI sites (EcoRI and BamHI ends blunted) of pET3-d (Studier et al., 1990) to give the wild-type Sar1p-expression plasmid, pANY3-14. To make the mutant version of Sar1p, nucleotides GAT (356–358) were converted to GTT in pANY3-5 by site-directed mutagenesis, which will yield the Asp-73 to Val point mutation ($Sar1p^{D73V}$). The 0.6-kb NcoI-EcoRI fragment with the mutation was transferred to the expression vector pMYE2 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 pMYE3-3. 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 *SAR1* expression plasmid (pANY3-14 or pMYE3-3) 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)[1] was added at the final concentration of 1 mM to invoke induction of *SAR1* 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 nM [α-^{32}P]GTP (74 kBq/ml) in 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, and 0.3% Tween20.

Partial Purification of Sar1p from E. coli Lysate

When the expression of *SAR1* was fully induced in *E. coli*, the product protein showed a tendency to form aggregates. Sar1p 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 pANY3-14 and pMYE3-3 were grown in 250 ml LB medium supplemented

1. *Abbreviation used in this paper*: IPTG, isopropyl-1-thio-β-D-galactoside.

with 50 μg/ml ampicillin at 37°C and induced for Sar1p 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). The mixture was let sit for 15 min on ice and centrifuged in a tabletop ultracentrifuge (model CP100H; Hitachi Ltd., Tokyo, Japan) (350,000 *g*, 30 min, 4°C). 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 Sar1p 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.

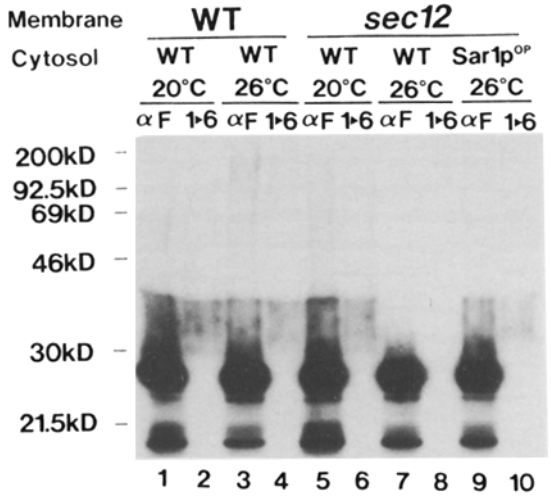

Figure 1. sec12 semi-intact cells show temperature-sensitive ER-to-Golgi transport in vitro which can be suppressed by cytosol containing excess Sar1p. The wild-type (*WT*) or *sec12* semi-intact cells (*Membrane*), ^{35}S-labeled prepro-α-factor, and the wild-type cytosol were incubated in the reaction buffer at 20 or 26°C for 60 min (see *Materials and Methods* for details). Cytosol (30 μg) from the cells overproducing Sar1p (*Sar1p^{OP}*) was also added to the reaction mixture at the beginning of the incubation (lanes *9* and *10*). The 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 anti-α-factor (*αF*) or anti-α1→6 mannose (*1→6*) 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.

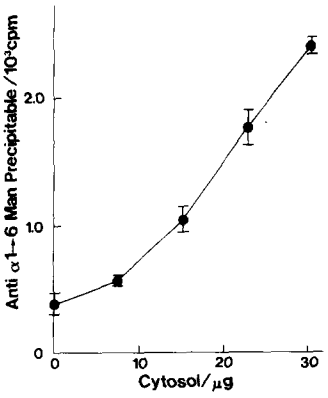

Figure 2. Dose dependence on cytosol from Sar1p overproducer of the suppression of *sec12^{ts}*. The *sec12* semi-intact cells, [^{35}S]prepro-α-factor and wild-type cytosol were incubated with the indicated amount of cytosol from the Sar1p overproducer at 26°C for 60 min. The Golgi form produced was 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.

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 (*sec12-4^{ts}*) 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, ^{35}S-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 *sec12^{ts}* 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 Sar1p Overproducer

With the temperature-sensitive reaction of the *sec12* semi-intact cells in hand, we proceeded to the analysis of Sar1p, the suppressor of *sec12^{ts}*. We have recently shown that Sar1p is tightly associated with membranes and only a small pool is detected in the wild-type cytosol (Nishikawa and Nakano, 1991). However, when the *SAR1* gene is overex-

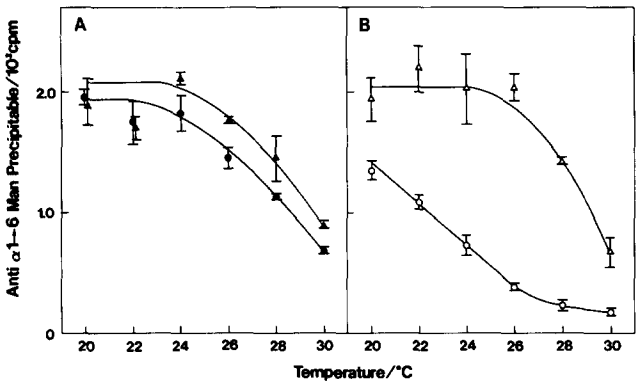

Figure 3. Temperature-dependence profiles of wild-type and *sec12* semi-intact cells with or without Sar1p-cytosol. The wild-type (*A*) or *sec12* (*B*) semi-intact cells, [^{35}S]prepro-α-factor, and wild-type cytosol were mixed with (▲, △) or without (●, ○) 60 μg cytosol from the Sar1p overproducer 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→6 Man antibody and scintillation counting.

pressed by the *GAL1* promoter, the cytosolic level of Sar1p is highly elevated. Using this cytosol from the Sar1p overproducer (hereafter referred to as Sar1p-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 Sar1p-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 Sar1p-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 Sar1p.

Temperature Dependence of Transport

The in vitro suppression of *sec12*^{ts} 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 Sar1p level; the amount of Sar1p was almost the same in the wild-type and *sec12* semi-intact cells as estimated by immunoblotting (not shown).

When the Sar1p-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 Sar1p-cytosol was observed for the wild-type semi-intact cells at all temperatures (Fig. 3 *A*, *solid triangles*). With the Sar1p-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 Sar1p-cytosol, suggesting that the *sec12* membranes are partially defective even at this low temperature. Such suppression effect was not observed below 16°C (not shown).

Expression of Sar1p in E. coli as a GTP-binding Protein

To unambiguously demonstrate that it is Sar1p that suppresses *sec12*^{ts}, we decided to make Sar1p in *E. coli*. The wild-type cDNA clone of *SAR1* (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 *SAR1* is regulated by the T7 promoter while the T7 polymerase is under the *lac* promoter control, so that the synthesis of Sar1p 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 *SAR1*-expression plasmid (Fig. 4 *A*, lane *4*, *arrowhead*). This species is in fact Sar1p as proved by immunoblotting with the anti-Sar1p antibody (Fig. 4 *B*, lane *8*). A small amount of Sar1p 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 *SAR1* 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 Sar1p indeed binds GTP, a GTP-blotting experiment was conducted for these *E. coli* lysates (Fig. 4 *C*). The lysates of the IPTG-induced cells harboring the *SAR1*-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-^{32}P-labeled GTP (Fig. 4 *C*, lanes *9* and *10*), [α-^{32}P]-ATP (lanes *11* and *12*), or [α-^{32}P]-GTP in the presence of 30-fold excess cold ATP (lanes *13* and *14*), washed, and autoradiographed. The result clearly indicates that Sar1p does bind GTP and the binding is specific to GTP; ATP could not be bound to Sar1p nor did it effectively compete with GTP binding. Thus Sar1p was efficiently produced in *E. coli* with the capability of GTP-specific binding.

Partial Purification of Wild-type and Mutant Sar1p Made in E. coli

The *E. coli* lysate containing Sar1p was tested for the ability to suppress *sec12*^{ts} 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 Sar1p 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 Sar1p 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 Sar1p 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-

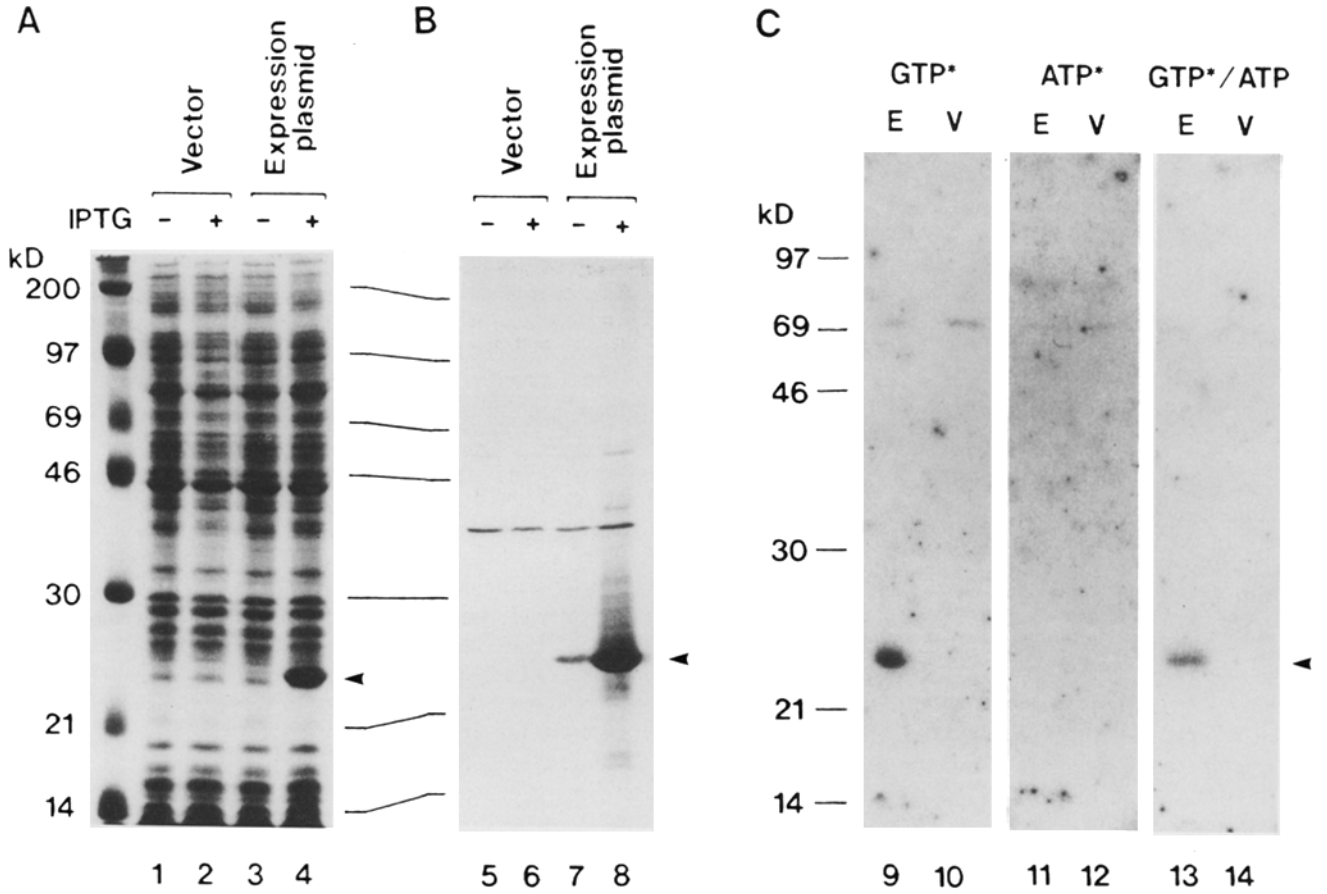

Figure 4. Expression of Sarlp in *E. coli* and GTP-binding analysis. The *E. coli* cells harboring either the *SAR1* expression plasmid (pANY3-14) or the vector alone (pET3-d) were incubated with or without 1 mM IPTG for 60 min. The cells were lysed with 2% SDS and subjected to SDS-PAGE. Gels were stained 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-^{32}P]GTP (*GTP**), 0.66 nM [α-^{32}P]ATP (*ATP**), or 0.66 nM [α-^{32}P]GTP plus 20 nM cold ATP (*GTP*/ATP*). Arrowheads indicate Sarlp.

metric scanning, the purity of Sarlp in the partially purified fraction was estimated to be ~30%.

We have been trying to construct a variety of *sar1* 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. 5 *A*, 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. 5 *B* 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. 5 *B*, 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^{ts}

The Sarlp fractions partially purified from *E. coli* lysates were assayed for the activity of suppressing *sec12^{ts}*. 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 Sarlp^{D73V} did not show the suppression activity at all (Fig. 6, *solid triangles*). Fig. 7 *A* shows that the *E. coli* wild-type Sarlp fraction in fact gave rise to the smeary Golgi form of pro-α-factor whereas the Sarlp^{D73V} fraction did not (Fig. 7 *A*, compare lanes *3* and *4*). The 26-kD ER form was normally produced in the presence of Sarlp^{D73V} (Fig. 7 *A*, 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^{ts}* 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^{ts}* (not shown). It is pos-

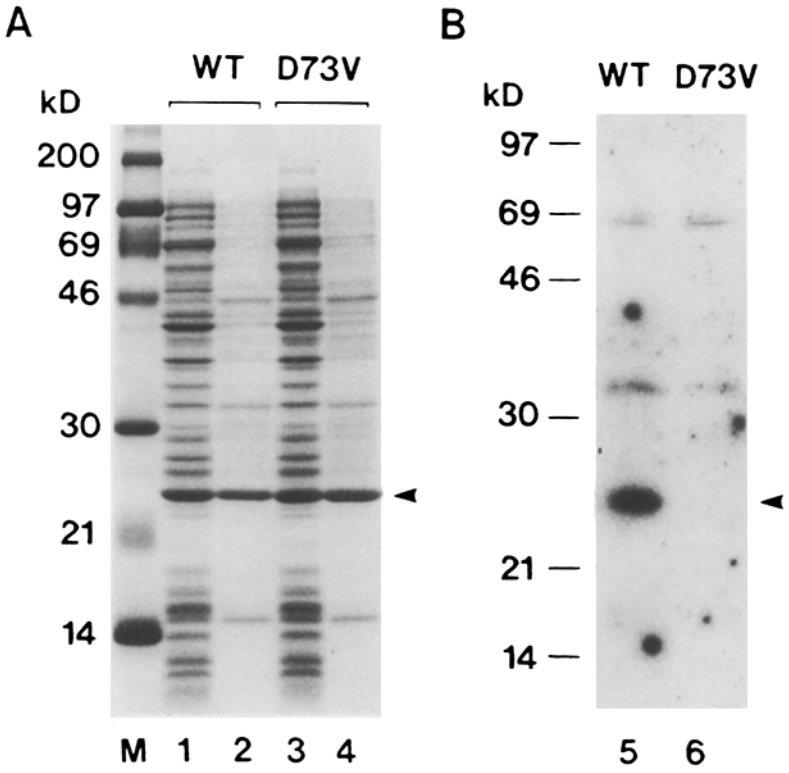

Figure 5. Partial purification of wild-type and mutant Sarlp from *E. coli* lysate. *E. coli* lysates were prepared from cells that were induced for the wild-type (*WT*) or mutant (*D73V*) Sarlp synthesis and subjected to partial purification of Sarlp as described in Materials and Methods. (*A*) SDS-PAGE and Coomassie blue staining of the total *E. coli* lysate (lanes *1* and *3*) and the partially purified samples (lanes *2* and *4*). (*B*) [^{32}P]GTP-blotting analysis of the total *E. coli* lysates. An autoradiogram with a purposely long exposure is presented to show the complete absence of GTP-binding of Sarlp^{D73V}. Arrowheads point to Sarlp.

sible that the mutant Sarlp^{D73V} 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*^{ts} 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 GTPγS, the non-hydrolyzable analogue of GTP. The wild-type Sarlp was renatured in the presence of GTPγS and dialyzed against the buffer containing GTPγS (Sarlp·GTPγS). As shown in Table I, the temperature sensitivity of the *sec12* semi-intact cells was not suppressed by this Sarlp·GTPγS (see also Fig. 7 *B*, lane *7*). In the experiments of Table I and Fig. 7 *B* all reactions contained free GTPγS at 0.94 and 1.1 μM respectively. Although GTPγS 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 GTPγS showed only a marginal effect on the ER-to-Golgi transport. Even in the presence of 1.1 μM GTPγS, the cytosol from the Sarlp-overproducing yeast completely suppressed *sec12*^{ts} (Fig. 7 *B*, lane *8*), indicating that the deficiency of suppression was due to the defect of Sarlp·GTPγS itself. These results suggest that the hydrolysis of GTP is required for the action of Sarlp in *sec12*^{ts} suppression.

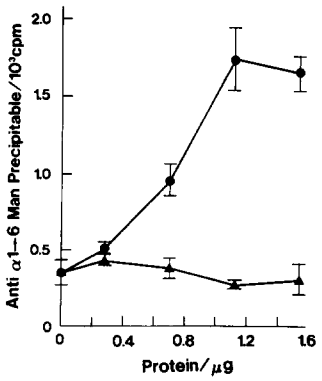

Figure 6. Suppression of *sec12*^{ts} by Sarlp produced in *E. coli*. The *sec12* semi-intact cells, [^{35}S]prepro-α-factor and the wild-type yeast cytosol (200 μg) were incubated with the indicated amount of the partially purified *E. coli* wild-type Sarlp fraction (●) or the mutant Sarlp^{D73V} fraction (▲) at 26°C for 60 min. The production of the pro-α-factor Golgi form was analyzed by immunoprecipitation with anti-α1→6 Man antibody. All reactions contained 11 μM free GDP as a carry-over from the *E. coli* fraction, which must have been converted to GTP by the ATP-regeneration system. The radioactivity of the immunoprecipitates assayed without the *E. coli* Sarlp fraction at 20°C was 1,690 ± 126 cpm.

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*^{ts} are temperature sensitive in this transport reaction and that this defect is perfectly cured by the elevated level of Sarlp. Since *SAR1* 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 *SEC12* and *SAR1* 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-

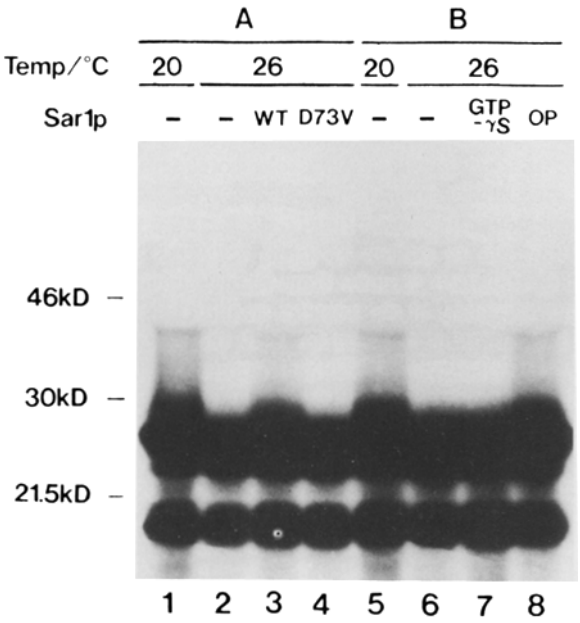

Figure 7. Evidence for the GTP-dependent suppression of *sec12*^{ts} by Sar1p. The *sec12* semi-intact cells, [^{35}S]prepro-α-factor and the wild-type yeast cytosol were incubated at 20 or 26°C for 60 min. Some reactions contained 1.1 μg of the *E. coli* fractions: Sar1p^{WT} (lane *3*), Sar1p^{D73V} (lane *4*), Sar1p^{WT}·GTPγS (lane *7*), or 30 μg yeast cytosol from the Sar1p overproducer (lane *8*). Reaction mixtures were subjected to immunoprecipitation with anti-α-factor antibody and analyzed by SDS-PAGE and fluorography. (*A*) The *E. coli* fractions (lanes *3* and *4*) were renatured in the presence of 1 mM GDP and dialyzed against 0.1 mM GDP resulting in the carry-over of free GDP at the final concentration of 11 μM. The final change of the dialyzing buffer was added to other reactions to keep the concentration of the free nucleotide constant. GDP or GTP at this low concentration had no effect on either translocation or transport. (*B*) The wild-type Sar1p produced in *E. coli* was renatured and dialyzed in the presence of GTPγS (lane *7*). As in *A*, the final concentration of free GTPγS was kept constant in all reactions (1.1 μM) by adding the last change of the dialyzing buffer.

uct level (Nakano et al., 1988; Nakano and Muramatsu, 1989; Nishikawa and Nakano, 1991). However, many efforts to demonstrate the physical interaction between the two proteins such as by coimmunoprecipitation or cross-linking have not been successful, suggesting that the interaction is transient in the process of vesicular transport. The in vitro suppression by Sar1p of the defect of the *sec12*^{ts} membranes will provide a useful system to investigate the nature of their interaction in biochemical ways. Although Sar1p is mostly associated with membranes in yeast cells, the functional Sar1p molecules can be supplied in a soluble form either from cytosol of the yeast cells overproducing Sar1p or by guanidine solubilization of Sar1p produced as insoluble aggregates in *E. coli*. We have preliminary evidence that soluble Sar1p is converted to the membrane-bound form during the in vitro reaction. When and how the molecule elicits membrane association will be an important question to understand the mechanistic function of Sar1p.

Independently, d'Enfert et al. have developed an in vitro assay which also requires the Sar1p function (d'Enfert et al., 1991). In their assay, conversion of the ER-form α-factor precursor to the Golgi form in microsome fractions is inhibited by the addition of microsomes containing overproduced Sec12p and this inhibition is released by excess Sar1p. Taken together, it is very likely that the interaction between Sec12p and Sar1p plays a key role in the transport from the ER to the Golgi apparatus.

Table I. Sar1p·GTPγS Cannot Suppress sec12^{ts}

Temperature	*E. coli* Sar1p	Anti-α1→6 Man precipitable GDP*	GTP*	GTPγS‡
	1.3 μg		*cpm*	
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.
* *E. coli* Sar1p 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 Sar1p·GDP (or Sar1p·GTP) (+) was added to the reaction, which gave the final concentration of free GDP [or GTP] at 11 μM.
‡ The same procedure was repeated as above except that 1 mM and 0.01 mM GTPγS was present during renaturation and the last dialysis, respectively, and the final concentration of free GTPγS in the reaction was 0.94 μM.

Sar1p as a GTP-Binding Protein

That Sar1p belongs to the GTP-binding protein superfamily has been deduced from the marked conservation of GTP-binding consensus motifs (Nakano and Muramatsu, 1989). In this paper we have demonstrated by *E. coli* expression that Sar1p in fact binds GTP and the binding is specific to GTP. We realized during the course of Sar1p purification from *E. coli* lysates that GTP or GDP is required for restoration of the Sar1p activity. When Sar1p that was solubilized with guanidine-HCl was subjected to renaturation by dilution and dialysis in the absence of GTP or GDP, no activity to promote transport was detected in vitro. This implies that guanine nucleotides are important for the Sar1p molecule to attain correct conformation.

In an attempt to analyze Sar1p function in vivo and in vitro, we have made several mutant versions of *SAR1* by site-directed mutagenesis (Yamagishi, M., S. Nishikawa, T. Oka, and A. Nakano, unpublished data). *SAR1*^{D73V} is one of such mutants; the product has the aspartate residue at position 73 replaced by valine and is well-expressed in *E. coli*. This Asp-73 residue corresponds to Asp-57 in mammalian ras proteins and is perfectly conserved in the GTP-binding protein superfamily as the DXXG motif (Dever et al., 1987). X-ray crystallographic analyses have shown that this residue is directly or indirectly involved in Mg^{2+} coordination which is essential for GTP hydrolysis (Schlichting et al., 1990; Pai et al., 1990). Despite its universal importance, few studies have been done that examined the effect of amino acid replacement at this position. We have shown in this paper that Sar1p^{D73V} transferred to a nitrocellulose sheet does not bind GTP at all. This means that the Asp-73 residue in Sar1p is very important for binding of GTP. Furthermore, partially purified Sar1p^{D73V} proves to be unable to suppress *sec12*^{ts} in vitro. This may indicate that GTP binding is essential for Sar1p to fulfill its function. Since GTP binding appears to be necessary for correct renaturation of Sar1p as mentioned above, it is also possible that Sar1p^{D73V} is inactive just be-

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*^{ts} unambiguously indicates 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. Sarlp that is renatured in the presence of GTPγS instead of GTP or GDP shows only a little activity to suppress *sec12*^{ts} 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 farnesyl moiety by the action of farnesyl transferase (probably encoded by *DPRI/RAM1* and *RAM2* in yeast) (Schafer et al., 1990; Goodman et al., 1990). Ypt1 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 NH_2-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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