Inhibition of GTP Hydrolysis by Sarlp Causes Accumulation of Vesicles That Are a Functional Intermediate of the ER-to-Golgi Transport in Yeast

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Abstract. The SAR1 gene product (Sarlp), a 21-kD GTPase, is a key component of the ER-to-Golgi transport in the budding yeast. We previously reported that the in vitro reconstitution of protein transport from the ER to the Golgi was dependent on Sarlp and Sec12p (Oka, T., S. Nishikawa, and A. Nakano. 1991. J. Cell Biol. 114:671-679). Sec12p is an integral membrane protein in the ER and is essential for the Sarl function. In this paper, we show that Sarlp can remedy the temperature-sensitive defect of the sec12 mutant membranes, which is in the formation of ER-to-Golgi transport vesicles. The addition of Sarlp promotes vesicle formation from the ER irrespective of the GTP- or GTP\(\gamma\)S-bound form, indicating that the active form of Sarlp but not the hydrolysis of GTP is required for this process. The inhibition of GTP hydrolysis blocks transport of vesicles to the Golgi and thus causes their accumulation. The accumulating vesicles, which carry Sarlp on them, can be separated from other membranes, and, after an appropriate wash that removes Sarlp, are capable of delivering the content to the Golgi when added back to fresh membranes. Thus we have established a new method for isolation of functional intermediate vesicles in the ER-to-Golgi transport. The sec23 mutant is defective in activation of Sarl GTPase (Yoshihisa, T., C. Barlowe, and R. Schekman. 1993. Science (Wash. DC). 259:1466-1468). The membranes and cytosol from the sec23 mutant show only a partial defect in vesicle formation and this defect is also suppressed by the increase of Sarlp. Again GTP hydrolysis is not needed for the suppression of the defect in vesicle formation. Based on these results, we propose a model in which Sarlp in the GTP-bound form is required for the formation of transport vesicles from the ER and the GTP hydrolysis by Sarlp is essential for entering the next step of vesicular transport to the Golgi apparatus.

In the secretory pathway, transport of proteins between successive compartments is mediated by small vesicles (Palade, 1975). Vesicles bud from the membrane of the donor compartment and specifically fuse with that of the acceptor. Characterization of carrier vesicles that mediate such intercompartmental transport has been one of the most important aspects of the studies on vesicular traffic. The transport from the ER to the Golgi apparatus represents the first vesicular step in the pathway. Many attempts have been made to isolate transport vesicles connecting these two organelles from mammalian and yeast cells (Lodish et al., 1987; Paulik et al., 1988; Groesch et al., 1990; Rexach and Schekman, 1991; Segev, 1991; Franzusoff et al., 1992; Lian and Ferro-Novick, 1993).

A non-hydrolyzable analogue of GTP, guanosine 5'-O-(3-thiotriphosphate) (GTP\(\gamma\)S), inhibits various steps of vesicular traffic including the transport from the ER to the Golgi (Baker et al., 1988; Ruohola et al., 1988; Beckers and Balch, 1989) and between distinct cisternae of the Golgi apparatus (Melançon et al., 1987). To date, a large number of low-molecular-weight GTPases have been shown to function in these events of vesicular transport (see Balch, 1990; Pfeffer, 1992). In the yeast secretory pathway, YPT1 and SEC4, which are similar to each other, are required for fusion of vesicles with the target membrane in the ER-to-Golgi and the Golgi-to-plasma membrane transport steps, respectively (Segev et al., 1988; Schmitt et al., 1988; Salminen and Novick, 1987). A number of homologous genes called nab family are implicated in various steps of vesicular fusion in mammalian cells.

We have identified a distinct type of small GTPase, Sarlp, which is essential for the ER-to-Golgi transport (Nakano and Muramatsu, 1989). We originally isolated the SAR1 gene as a multicopy suppressor of the sec12 mutation (Nakano et al., 1988). The SEC12 gene has been shown to code for a type-II integral membrane protein, which is almost exclusively localized in the ER (Nakano et al., 1988; d'Enfert et al., 1991a; Nishikawa and Nakano, 1993). Sarlp is also mostly...
localized on the ER membrane (Nishikawa and Nakano, 1991). In vivo and in vitro analyses have indicated that the interaction between Sarlp and Sec12p is critical for their functions (Nakano and Muramatsu, 1989; Oka et al., 1991; d'Enfert et al., 1991b). Evidence has also been presented that these proteins are involved in formation of vesicles from the ER (Kaiser and Schekman, 1990; d'Enfert et al., 1991b; Rexach and Schekman, 1991).

In this paper, we show that in our cell-free assays Sarlp-GTP γS suppresses the sec12 defect in vesicle formation but is incapable of promoting transport to the Golgi, thus causing accumulation of vesicles. These vesicles can be chased to the Golgi if they are washed and added back to fresh membranes. Similar accumulation of vesicles are also seen when the GTP hydrolysis is inhibited by the sec23 mutation in the presence of excess Sarlp. The functional roles of Sarlp in the early events of ER-to-Golgi transport, namely budding and release of transport vesicles from the ER membrane will be discussed.

Materials and Methods

Strains
The yeast strains used in this study were X2180-IA (mal gal2 CUP1 MATa) (Yeast Genetic Stock Center, Berkeley, CA), MBY10-7A (sec23-4 ura3-52 leu2-3, 112 trpl-289 his3 his4 suc gal2 MATa) (Nakano et al., 1988), and MBY8-20C (sec23-1 ura3-52 leu2-3, 112 trpl-289 his3 his4 gal2 MATa) (Hicke and Schekman, 1989). The cells were grown at 30 or 24°C (for sec mutants) in YPD medium (2% peptone [Nihon Pharmaceutical Co. Ltd., Tokyo, Japan], 1% yeast extract [Difco Laboratories, Inc., Detroit, MI] and 2% glucose).

In Vitro Transport Reaction
[35S]-Labeled prepro-α-factor was translated in vitro in a yeast translation lysate as described (Oka et al., 1991). Semi-intact cells and cytosol were prepared from the wild-type and sec mutant cells according to the method of Baker et al. (1988).

One-step standard transport reaction was performed as described (Oka et al., 1991). To investigate the temperature-sensitive defect of sec23 which is borne in both membranes and cytosol, we devised a two-step transport reaction as follows. Semi-intact cells prepared from the sec23 mutant were incubated at 17°C for 17 min with 60 μg of cytosol containing labeled prepro-α-factor and ATP-regeneration system (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). The cells were grown at 30 or 24°C (sec mutants) and loaded on the top of 15-45% (wt/vol) linear sucrose gradient buffered (20 mM Hepes-KOH [pH 6.8], 150 mM KOAc and 5 mM Mg(OAc)2). The pellet was resuspended in 55% (wt/vol) glycerol solution (in 20 mM Hepes-KOH [pH 6.8], 150 mM KOAc and 5 mM Mg(OAc)2). The pellet was resuspended in 55% (wt/vol) glycerol solution, placed at the bottom of an ultracentrifuge tube and overlaid with 0.84 ml each of 50, 45, 40, and 35% (wt/vol) sucrose. The gradient was centrifuged at 120,000 g for 60 h in Hitachi RPS65T rotor and fractionated. The distribution of vesicles was analyzed as above.

To demonstrate the presence of Sarlp on the vesicles, we employed the centrifugal field gradient. The S-20 fraction from the reaction accumulating vesicles was centrifuged at 100,000 g for 60 min through a 15-45% (wt/vol) sucrose cushion. The pellet was resuspended in 1% SDS and analyzed by immunoblotting with the anti-prepro-α factor antibody, SDS-PAGE, and fluorography.

Chase Reaction of Intermediate Vesicles to the Golgi

Semi-intact cells were incubated with Sarlp-GTP γS for 60 min as in the standard transport reaction and spun down by 20,000 g centrifugation for 1 min. The supernatant fraction (S-20) was further centrifuged at 100,000 g for 30 min through 1 mM sorbitol cushion. The pellet was resuspended in the reaction buffer and reincubated with cytosol, ATP-regeneration system and fresh semi-intact cells at 17 or 27°C for 60 min. The transport of pro-α-factor to the Golgi apparatus was analyzed using the anti-α-1,6 mannosyl linkage antibody as described above.

Density Gradient Analysis of Vesicles

The S-20 fraction containing intermediate vesicles was centrifuged at 100,000 g for 60 min through 15% (wt/vol) sucrose cushion. The pellet was resuspended in a sucrose solution at the final sucrose concentration of 10%, and loaded on the top of 15-45% (wt/vol) linear sucrose gradient buffered by 20 mM Hepes-KOH (pH 6.8) in a 5-ml ultracentrifuge tube. The gradient was centrifuged at 120,000 g for 140 min in Hitachi RPS65T rotor and fractionated from the bottom of the tube. The fractions were subjected to precipitation by 10% TCA. The precipitates were dissolved in 1% SDS and analyzed by immunoprecipitation with the anti-prepro-α factor antibody, SDS-PAGE, and fluorography.

The vessel fractions containing the ER-form of pro-α-factor in this velocity sucrose gradient were further subjected to an equilibrium flotation analysis. The peak fractions were mixed with concentrated sucrose to make the final concentration 50% (wt/vol), placed at the bottom of a 5-ml ultracentrifuge tube and overlaid with 0.67 ml each of 45, 40, 35, 30, 25, and 20% (wt/vol) sucrose. The gradient was centrifuged at 120,000 g for 46.5 h in RPS65T rotor and fractionated from the bottom. The fractions were subjected to precipitation by TCA. The precipitates were dissolved in 1% SDS and analyzed by immunoprecipitation with the anti-Sarlp antiserum. The ER-form of pro-α factor in each fraction was analyzed by immunoblotting with the anti-prepro-α factor antibody or measured in the blot by Image analyzer bass-2000 (Fujifilm Co. Ltd., Tokyo, Japan).

Results

The Defect of the sec12 Semi-intact Cells

Is in the Step of Vesicle Formation from the ER and Is Suppressed by Sarlp-GTP γS

Our previous work (Oka et al., 1991) indicated that the sec12 semi-intact cells have a temperature-sensitive defect in the ER-to-Golgi transport that is remedied by the addition of excess Sarlp. To further specify the steps requiring the functions of Sec12p and Sarlp, formation of vesicles from the ER was quantified. The sec12 semi-intact cells were incubated at the restrictive temperature, 26°C, in the presence and absence of additional Sarlp. The reaction mixture was briefly centrifuged (20,000 g, 1 min) to bring down the semi-intact cells containing large cellular organelles. The resulting supernatant (S-20) was treated with trypsin. The trypsin-resistant ER-form of pro-α-factor was quantified as vesicles released from the ER.

As shown in the bottom panel of Fig. 1 A, only a very small amount of the ER-form (arrow head) was released into the vesicle fraction in the absence of Sarlp, confirming the observation of Rexach and Schekman (1991) that the sec12 membrane is defective in the formation of vesicles. Fig. 1 C
**Figure 1.** Sarlp-GTPγS suppresses the sec12 defect in vesicle formation but blocks the transport to the Golgi apparatus. (A) The sec12 semi-intact cells were incubated at 26°C in a standard cell-free transport reaction with 2 μg Sarlp-GTP, with 2 μg Sarlp-GTPγS, or without any additional Sarlp. At times indicated, an aliquot of the reaction mixture was centrifuged at 20,000 g for 1 min and the supernatant fraction (S-20) was treated with trypsin and then subjected to immunoprecipitation with anti-prepro-α-factor antibody, SDS-PAGE, and fluorography. For details, see under Materials and Methods. Arrow heads indicate the ER-form of pro-α-factor. (B) The same experiment as A was quantified by scintillation counting for vesicles formed (Con A-precipitable counts; left) and for transport to the Golgi apparatus (immunoprecipitation with anti-α1→6 mannose antibody; right). (o) sec12 membranes plus Sarlp-GTP, (●) sec12 membranes plus Sarlp-GTPγS, (△) sec12 membranes without Sarlp. (C) Temperature dependence profiles of the sec12 membranes. The sec12 semi-intact cells were incubated for 60 min at the indicated temperatures. After reaction, the S-20 fraction was treated with trypsin and subjected to precipitation with Con A-Sepharose (●) or with anti-α1→6 mannosyl linkage antibody (○).
Sarlp-GTP\(_{\text{Y}}\)S accumulates vesicles in a dose-dependent manner. The wild-type (WT) and sec12 semi-intact cells were incubated for 60 min with the indicated amount of Sarlp-GTP\(_{\text{Y}}\)S at 20 and 26°C, respectively. After reaction, S-20 fraction was prepared as in Fig. 1 A and analyzed by immunoprecipitation with anti-prepro-\(\alpha\)-factor antibody.

The addition of Sarlp-GTP completely suppressed this defect of sec12 (Fig. 1 A, top panel). Fig. 1 B shows the quantification of the same experiment as Fig. 1 A. Sarlp-GTP restored the ability of the sec12 membrane to form vesicles (Con A-precipitable pro-\(\alpha\)-factor) at 26°C to the level of the permissive temperature (compare with Fig. 1 C). Furthermore, the incubation with Sarlp-GTP gave rise to the Golgi-form of pro-\(\alpha\)-factor (Fig. 1 A; smeary form extending above the ER-form), which is probably due to the Golgi membranes released in the vesicle fraction. Thus the increased level of Sarlp-GTP could cure the sec12 defect of vesicle formation and promote the overall transport to the Golgi.

Interestingly, when the sec12 membranes were incubated with Sarlp-GTP\(_{\text{S}}\) (Fig. 1 A, middle panel), the ER-form of pro-\(\alpha\)-factor was liberated into the vesicle fraction as efficiently as in the case of Sarlp-GTP. However, Sarlp-GTP\(_{\text{S}}\) was unable to promote the targeting of vesicles to the Golgi at all (see closed circles in Fig. 1 B). This clearly indicates that the GTP hydrolysis by Sarlp is not essential for vesicle formation but is required for proceeding to the subsequent steps of transport. It should be noted here that the total...
amount of vesicles produced by Sarlp-GTPγS (Con A-precipitable counts) is appreciably smaller than the case with Sarlp-GTP. This may be because the vesicle formation reaction plateaued earlier during the time course when the GTP hydrolysis was inhibited.

As shown in Fig. 2 (lanes 6–10), the release of vesicles from the sec12 membrane increased in proportion to the amount of the added Sarlp-GTPγS, indicating that it is the active form of Sarlp that was limiting in the reaction. Sarlp-GTPγS showed a transdominant effect to the wild-type membranes as well; it blocked the formation of the Golgi-species but did not reduce the ER-form (Fig. 2, lanes 1–5). Again the consequence of the inhibition of GTP hydrolysis was the blockade of vesicle targeting to the Golgi, not of vesicle formation.

**Characterization of Vesicles Accumulated by Sarlp-GTPγS**

The vesicles generated by incubation of the sec12 semi-intact cells with Sarlp-GTPγS were analyzed by sedimentation through a linear density gradient of sucrose. The migration of the vesicles was assessed by assaying fractions for the ER-form of pro-α-factor. As shown in Fig. 3 A, the vesicles containing the ER-form emerged virtually as a single peak at the density of 29% (wt/vol) sucrose. This peak defines the vesicles accumulated by Sarlp-GTPγS, because the appearance of this peak was dependent on the added Sarlp-GTPγS (see below). One may also note a very small peak at fraction 8. As the density of this fraction 38% (wt/vol) coincides with the reported value of the yeast ER membrane (Goud et al., 1988), it probably represents small fragments of the ER escaped into the S-20 fraction. Obviously the vesicles accumulating in this experiment are quite distinct from such ER remnants.

The peak fractions at 29% (wt/vol) sucrose of this sedimentation centrifugation were further analyzed on an equilibrium density gradient by flotation. As shown in Fig. 3 B, the vesicles were equilibrated as a single peak at the density of 37% (wt/vol) sucrose (1.171 g/cm³). This suggests that the vesicles accumulated by Sarlp-GTPγS is a reasonably homogeneous population of membranes.

**Vesicles Accumulated by Sarlp-GTPγS Are a Functional Intermediate of the ER-to-Golgi Transport**

Are these vesicles produced by Sarlp-GTPγS a true intermediate of the ER-to-Golgi transport? In some cases, vesicles accumulated by the action of GTPyS seemed to be a dead end product of the reaction and were not able to proceed to further steps of transport (Melaço et al., 1987; Rexach and Schekman, 1991). In fact, the ER-form of pro-α-factor in the S-20 fraction did not acquire the Golgi modification when mixed with fresh semi-intact cells (data not shown). To address this problem, we tried to wash the accumulating vesicles extensively. We first found that when the vesicles were washed with 2 M urea, they restore the ability to be chased to the Golgi apparatus. Meanwhile it turned out that washing through a sorbitol cushion was enough for the chase reaction to work. Sucrose was not suitable for washing because it was found to inhibit the transport reaction at as low as 1%. Fig. 4 shows the transport reaction using the sorbitol-washed vesicles and fresh semi-intact cells. The vesicles released into the S-20 fraction from the semi-intact cells in the presence of Sarlp-GTPγS were spun through 1 M sorbitol cushion, resuspended in the transport reaction buffer, mixed with cytosol and ATP regeneration system (ATP) and fresh wild-type semi-intact cells, and further incubated at 20°C for 60 min. The chase of the vesicles to the Golgi was measured by immunoprecipitation with anti-α1→6 mannose antibody. Among 4,700 cpm of pro-α-factor present in the S-20 fraction, 755 cpm (left panel) and 805 cpm (right panel) acquired the α1→6 mannose linkage in the complete reactions. Additions to the second incubation were: anti-Yptlp antibody (αYptlp Ab), 4 μg; (Yptlp) 6 μg; (Sarlp-GTP); 7.2 μg; and (Sarlp-GTPγS) 7.2 μg. (B) Chase of the vesicles to the Golgi in the sec12 semi-intact cells. Vesicles generated by Sarlp-GTPγS were isolated by centrifugation as described in A and incubated for 60 min at 17 or 27°C with the wild-type or sec12 semi-intact cells.

**Figure 4.** Vesicles accumulated by Sarlp-GTPγS are a functional intermediate of the ER-to-Golgi transport. (A) The wild-type semi-intact cells were incubated with Sarlp-GTPγS at 20°C for 60 min. The S-20 fraction was prepared and centrifuged at 100,000 g for 30 min through 1 M sorbitol cushion. The pellet was resuspended in the transport reaction buffer, mixed with cytosol, ATP-regeneration system (ATP), and fresh wild-type semi-intact cells (Membranes), and further incubated at 20°C for 60 min. The chase of the vesicles to the Golgi and the added semi-intact cells. Vesicles generated by Sarlp-GTPγS were isolated by centrifugation as described in A and incubated for 60 min at 17 or 27°C with the wild-type or sec12 semi-intact cells.
the chase reaction. To rule out this possibility, the chase was performed with the sec12 semi-intact cells at 17 and 27°C. As shown in Fig. 4 B, the sec12 membranes were as active as the wild type at both temperatures, indicating that the chase reaction is not blocked by the sec12 lesion any more. This experiment not only rules out the suspected bypass during the chase but also suggests that the function of Sec2lp is not required in the later steps of transport.

These results have led us to conclude that the vesicles accumulated by Sarlp-GTPyS are a functional intermediate of the ER-to-Golgi transport. We have also established a method to dissect this vesicular transport process into two steps: the early one requiring Sec12p and Sarlp and the late one dependent on Yptlp.

**Sarlp Is Present on the Intermediate Vesicles**

All the results hitherto described suggest that Sarlp has to fulfill its GTPase activity after vesicles are completed and released from the ER. Since the GTP hydrolysis by Sarlp is essential for entering subsequent reactions of vesicular targeting to the Golgi, it would be most reasonable that Sarlp functions on the vesicles. However, despite strenuous efforts we could not prove the presence of Sarlp in the vesicle fractions of the sucrose density gradient. Fig. 3 C shows one of such analyses; Sarlp was detectable by immunoblotting in the S-20 fraction but not at all in the vesicle fraction prepared by sucrose density gradient centrifugation. It is known that high concentration of sucrose sometimes affects assembly of protein complexes and therefore it is possible that Sarlp falls off from the vesicles during centrifugation. Indeed, the fact that washing the vesicles through 1 M sorbitol restores the competence of transport supports this possibility. So we decided to try density gradient of glycerol, which probably has a less deleterious effect on protein assembly. Fig. 5 shows the result of a flotation analysis of the vesicles through a 25–55% (wt/vol) glycerol density gradient. The vesicles containing the ER-form migrated as a single peak at the density of 41% (wt/vol) glycerol (Fig. 5 B, open circles). When the S-20 fraction was prepared from the sec12 membranes incubated without Sarlp-GTPyS, this peak was not observed at all (Fig. 5 C, open circles). The glycerol gradient fractions generated by the incubation with Sarlp-GTPyS were analyzed by immunoblotting using the anti-Sarlp antibody. As shown in Fig. 5 A (also in closed circles in Fig. 5 B), Sarlp clearly comigrated with the peak of pro-α-factor, indicating that Sarlp is present on the vesicles. This supports the view that Sarlp executes its GTPase activity on the vesicles.

**Sarlp-GTPyS Suppresses the sec23 Defect in Vesicle Formation**

Recently Yoshihisa et al. (1993) have found that the SEC23 gene product has an activity as a GTPase-activating protein (GAP) toward Sarlp, which is deficient in the sec23 mutants. In vivo and in vitro studies have indicated that the sec23 mutants have a defect in vesicle formation from the ER (Kaiser and Schekman, 1990; Resach and Schekman, 1991). These observations direct the role of GTP hydrolysis by Sarlp in the vesicle formation step. Is this inconsistent with our data described above? We reasoned that the block of GTP hydrolysis would hamper the cycling of Sarlp, perhaps between the vesicles and the ER membrane, and that this may eventually lead to the shortage of active Sarlp on the ER membrane and the shut off of the vesicle formation. In fact, in the vesicle formation assay using the sec12 semi-intact cells (see Fig. 1 B), Sarlp-GTPyS promoted the release of vesicles but it plateaued at a lower level than the case of Sarlp-GTP. A similar scenario might apply to the sec23 mutants.

We prepared semi-intact cells and cytosol from the sec23-1 mutant and tested the effect of Sarlp (Fig. 6). The reaction reconstituted from the sec23 membranes and cytosol showed a defect in vesicle formation at 27°C as previously reported (Rexach and Schekman, 1991). However, the extent of the temperature-sensitive block was only partial (ca. 50%) unlike the case of sec12. This is also noticeable in the data of Rexach and Schekman (1991). It could be due to the leakiness the sec23 allele used here, although sec23-1 is a
Figure 6. Sarlp suppresses the sec23 defect in vesicle formation. (A) The sec23 semi-intact cells were incubated with the indicated amounts of Sarlp-GTP at 17 or 27°C. To avoid the effect of the wild-type Sec23 protein present in the prepro-α-factor preparation, a two-step reaction procedure was employed (see Materials and Methods). The S-20 fractions were treated with trypsin and subjected to precipitation with Con A-Sepharose or with anti-α1→6 mannose antibody. (B) The sec23 semi-intact cells were incubated with 4.3 μg of Sarlp-GTP or Sarlp-GTPγS at 27°C for 60 min. S-20 fractions were analyzed for Con A-precipitable counts as in A.

reasonably tight allele in terms of temperature sensitivity of growth. Alternatively, the seeming defect in vesicle formation is a secondary effect of the blockade of Sarlp cycling as mentioned above. If the supply of Sarlp is limiting in the sec23 reaction due to the lesion in the cycling, the increase of Sarlp should remedy the defect. This was in fact the case. Exogenously added Sarlp-GTP suppressed the sec23 defect in a dose-dependent manner (Fig. 6 A). The added Sarlp did not have to be in the GTP-form. Sarlp-GTPγS was as effective as Sarlp-GTP in this suppression (Fig. 6 B). Since the GTP hydrolysis by Sarlp is required for transport of vesicles to the Golgi and it probably requires the Sec23p function, the excess Sarlp did not give rise to the Golgi-species even in the GTP-bound form (Fig. 6 A).

Figure 7. Overexpression of SARI partially suppresses the temperature-sensitive growth of the sec23 mutant. The sec23-I mutant cells (MBYB-20C) harboring SARI on a multicopy plasmid (pANY2-7) or the vector alone (pSEYS) (see Nakano and Muramatsu, 1989) were streaked on a YPD plate and incubated for 2 d at 33.5°C, a semi-restrictive temperature for sec23-I.

The suppression of the sec23 defect by Sarlp was also seen in vivo. At a semi-restrictive temperature, 33.5°C, at which the sec23 mutant cells scarcely grew on the YPD plate, the introduction of the SARI gene on a multicopy plasmid completely cured the defect (Fig. 7). Such suppression was not observed at a more restrictive temperature, 37°C (see Nakano and Muramatsu, 1989). This is probably because the overproduction of Sarlp may circumvent the cycling defect of sec23 but cannot remedy the GAP deficiency so that some residual activity of Sec23p is required to enable the transport to the Golgi.

Discussion

A large number of GTP-binding proteins or GTPases have been found to function in intracellular vesicular traffic. The vesicular transport from the ER to the Golgi apparatus in yeast provides an intriguing system, because two distinct small GTPases, Sarlp and Yptlp, are shown to play pivotal roles in budding and formation of intermediate vesicles and in targeting and fusion of them, respectively. In this paper, we have focused on the role of GTP hydrolysis in the function of Sarlp. The conclusion led from our results of cell-free assays is that the hydrolysis of GTP by Sarlp is not essential for vesicle formation but is required for entering the subsequent step. Sarlp-GTPγS accumulates vesicles that are a functional intermediate of the transport. The step of vesicle targeting to the Golgi appears to require release of Sarlp from the vesicles and is dependent on Yptlp.

Sarlp But Not Its GTPase Activity Is Required for Vesicle Formation

The cell-free system we are using is based on the fact that the temperature-sensitive defect of sec12 in ER-to-Golgi transport is suppressed by the elevated level of Sarlp (Nakano and Muramatsu, 1989; Oka et al., 1991). The defect of sec12 has been shown to lie in the step of vesicle formation from the ER (Kaiser and Schekman, 1990; Rexach
Sec12p is an integral membrane protein in the ER, whose cytoplasmic domain has recently been demonstrated to possess an activity to catalyze the GDP/GTP exchange of Sarlp (Barlowe and Schekman, 1993). The rescue of the sec12 defect by Sarlp is thus easily explained by the supplementation of the active form (i.e., GTP-bound form) of Sarlp.

We previously reported that Sarlp-GTPγS was unable to suppress the defect of sec12 in the overall ER-to-Golgi transport reaction (Oka et al., 1991). This was the first demonstration of the requirement of GTP hydrolysis by Sarlp. In the present study, however, we have found that the primary defect of sec12 in the formation of vesicles from the ER is suppressed by Sarlp in either the GTP- or GTPγS-bound form. Sarlp-GTPγS is able to promote formation of vesicles from the sec12 ER membrane efficiently. The amount of vesicles formed is completely dependent on the dose of Sarlp-GTPγS added, indicating that the active form of Sarlp is limiting in the sec12 membranes. It should be pointed out here that Sarlp-GTP is more active than Sarlp-GTPγS in the vesicle formation. This is probably because the hydrolysis of GTP enables the cycling of GTP- and GDP-bound states of Sarlp and thus drives turnover of the budding reaction. In fact, the vesicle production by Sarlp-GTP continues to increase whereas that by Sarlp-GTPγS plateaus in time course experiments (see Fig. 1B).

In contrast to the vesicle formation, Sarlp-GTPγS is totally inactive in proceeding to the subsequent step, i.e., targeting of the vesicles to the Golgi. Consequently, the vesicles formed by the action of Sarlp-GTPγS accumulate in the supernatant. Sarlp-GTPγS also shows a transdominant effect on the wild-type membranes. The step of blockage is again in the targeting of vesicles to the Golgi but not in the vesicle formation.

A similar defect is also seen with the sec23 mutant. Sec23p is a GTPase-activating protein of Sarlp (Yoshiihisa et al., 1993) and has been thought to function in the vesicle budding from the ER (Kaiser and Schekman, 1990; Rexach and Schekman, 1991). This appears to point the role of GTP hydrolysis by Sarlp to the vesicle formation step contrary to the above observations. However, we reexamined the defect of sec23 in our cell-free assay and revealed that the sec23 mutant has only a partial defect in vesicle formation that is again suppressed by Sarlp in a dose-dependent manner. In this suppression, Sarlp-GTPγS is as active as Sarlp-GTP; supporting our conclusion that the GTP hydrolysis by Sarlp is not essential for vesicle formation.

Why does the sec23 mutant show the budding defect then? We would suggest that it is due to the defect of Sarlp cycling between the vesicles and the ER. Presumably the GTPase activity is required for the release of Sarlp from the vesicles. The lesion in the GAP activity of Sec23p would then block the return of Sarlp to the ER, which eventually leads to the deficiency of the active form of Sarlp at the budding site of the ER. We will come back to this point later.

**Intermediate Vesicles of the ER-to-Golgi Transport**

The inhibition of vesicle targeting to the Golgi by Sarlp-GTPγS causes accumulation of vesicles in either wild-type or sec12 membranes. These vesicles can be isolated from other membranes by differential centrifugation and density gradient fractionation. If the vesicles are washed with 1 M sorbitol, they restore the ability of targeting to and fusion with the Golgi membrane. The chase of the washed vesicles to the Golgi requires ATP, cytosol, and the acceptor membranes. The antibody against Yptlp inhibits this chase reaction. Thus the vesicles accumulated by Sarlp-GTPγS retain properties as an intermediate of the ER-to-Golgi transport. Interestingly, the targeting of these vesicles to the Golgi is not affected by either the sec12 block or Sarlp-GTPγS. This suggests that functions of Sec12p and Sarlp are only required in the early events but not in the later targeting reactions.

**Function of Sarlp as a GTPase**

We were able to demonstrate that Sarlp itself is present on the vesicles accumulated by Sarlp-GTPγS. To show this, however, it was necessary to perform all the centrifugation procedures in glycerol. If the vesicles are spun through a high concentration of sorbitol or sucrose, they lose Sarlp and instead regain the targeting competence. These findings suggest that Sarlp fulfills its GTPase activity on the completed vesicles and that the release of Sarlp-GDP from the vesicles is required for the vesicle targeting to the Golgi.

This would predict that the vesicles prepared by glycerol gradient are inactive in the chase reaction if they keep Sarlp-GTPγS tightly bound on them. However, those vesicles show a low but significant activity of targeting to the Golgi (Oka, T., unpublished observations). This may suggest that a considerable fraction of Sarlp-GTPγS falls off from the vesicles even in the glycerol gradient. On the other hand, it provides another line of evidence that the vesicles accumulated by Sarlp-GTPγS represent a functional intermediate of the ER-to-Golgi transport.

Based on the data available, we propose a model of Sarlp function as shown in Fig. 8. Sec12p is located at the budding site of the ER membrane. Some signal that is unknown at present triggers interaction between Sec12p and Sarlp, which then converts Sarlp from the inactive GDP-form to the active GTP-form. Sarlp-GTP promotes budding, formation, and release of a vesicle with the help of other components (probably including the Sec13p complex [Pryer et al., 1993] and maybe coatamers [Hosobuchi et al., 1992]). Then Sec23p acts on Sarlp to activate GTPase. The block of GTP hydrolysis...
sis by either GTP\gamma S or the sec23 mutation would make Sarlp sitting on the vesicle, which prevents entering the next step of transport. If Sarlp is removed from the vesicle by normal GTP hydrolysis or by artificial washing with sorbitol, the vesicle acquires competence of targeting. The fact that readdition of Sarlp-GTP\gamma S does not inhibit the later reaction may indicate that the release of Sarlp provokes a substantial change in the properties of the vesicles. In this regard, it may be worth mentioning that the vesicles yielded by Sarlp-GTP\gamma S are slightly but reproducibly denser than the vesicles accumulated by the action of the anti-Yptlp antibody (Oka, T., unpublished observations). Whether they are coated would be an important question to be addressed.

There is another possibility that explains the present data. It could be the release of the completed vesicle from the ER that requires GTP hydrolysis. In this case, the active form of Sarlp promotes budding and completion of a vesicle but the block of GTP hydrolysis by GTP\gamma S or by the sec23 mutation should keep the vesicles staying on the ER membrane. Perhaps the brief centrifugation we perform after the in vitro reaction causes the detachment of the vesicles together with Sarlp. We think it is unlikely that interruption of such a weak association requires consumption of GTP, however further studies, especially a morphological examination of the reactions, will be necessary to test these possibilities.

We know that Sec12p is mostly residing in the ER but is also subject to sugar modifications in the Golgi (Nakano et al., 1988; d'Enfert et al., 1991a; Nishikawa and Nakano, 1993). At least some portion of Sec12p has to be in the vesicles. However, because Sec12p is a very low abundance protein, we could not detect any Sec12p in the vesicle fraction. The meaning of the transport of Sec12p to the Golgi remains to be elucidated. It should be also mentioned here that Sarlp is present on our vesicle fraction but is not highly enriched. Presumably only a catalytic amount of Sarlp is enough to execute its function on the vesicle. Alternatively, the vesicles we prepared might have lost a large part of the bound Sarlp as discussed above, even though they were isolated by glycerol gradient. It would be also interesting to examine whether other membrane proteins such as Boslp (Lian and Ferro-Novick, 1993) and Sec22p/Sly2p (Ossig et al., 1991; Newman et al., 1992) are present on the vesicles we prepared.

**Further Dissection of the ER-to-Golgi Transport**

The assay we have established provides a novel tool to dissect the ER-to-Golgi transport reactions especially in terms of differentiation of the roles of two distinct class of GTPases, Sarlp and Yptlp. They divide the work in the early and late steps of the transport, but could interact with each other. In fact, we have found that Yptlp is already present in the vesicle fraction produced by Sarlp-GTP\gamma S (Oka, T., unpublished observations). To understand the whole sequence of the cascade reactions in this complex process, it would be important to dissect the reactions further in combination with assays developed by other researchers. The identities and relationships of intermediate vesicles being characterized in different laboratories (Groesch et al., 1990; Rexach and Schekman, 1991; Segev et al., 1988; Franzusoff et al., 1992; Lian and Ferro-Novick, 1993) should be defined biochemically and morphologically. With regard to the function of Sarlp as a GTPase, genetic approach should also be very useful. We have recently obtained 3 temperature-sensitive and 7 dominant-negative alleles of SARI by random and site-directed mutagenesis (Nakano, A., Ohtsuka, M., Yamagishi, T., Yamanushi, K., Kimura, S., Nishikawa, and T. Oka, manuscript in preparation). The purification of these mutant proteins are now in progress using an E. coli expression system. The analysis of these SARI mutants in vivo and in vitro should provide us with further information on the role of a GTPase to prove or disprove the current models of vesicular transport.

We are grateful to Dieter Gallwitz for the anti-Yptlp antibody and the purified recombinant Yptlp, to Alex Franzusoff for a suggestion to use glycerol density gradient centrifugation, and to Charles Barlowe, Tohru Yoshihisa, and Randy Schekman for exchange of information before publication. We also thank Yasuhiro Anraku and Shuh-ichi Nishikawa for discussion and critical comments.

This work was supported by a research grant from the Human Frontier Science Program Organization, by a research fund from the Naito Foundation, and by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan. T. Oka was awarded by a predoctoral fellowship from the Japan Society for the Promotion of Science.

Received for publication 15 June 1993 and in revised form 15 November 1993.

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