Inhibition of GTP Hydrolysis by Sar1p 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 (Sar1p), 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 Sar1p 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 Sar1 function. In this paper, we show that Sar1p 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 Sar1p promotes vesicle formation from the ER irrespective of the GTP- or GTPγS-bound form, indicating that the active form of Sar1p 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 Sar1p on them, can be separated from other membranes, and, after an appropriate wash that removes Sar1p, 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 Sar1 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 Sar1p. 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 Sar1p in the GTP-bound form is required for the formation of transport vesicles from the ER and the GTP hydrolysis by Sar1p 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) (GTPγS)[1], 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 *rab* family are implicated in various steps of vesicular fusion in mammalian cells.

We have identified a distinct type of small GTPase, Sar1p, 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., 1991*a*; Nishikawa and Nakano, 1993). Sar1p is also mostly

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1. *Abbreviations used in this paper*: GAP, GTPase-activating protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

localized on the ER membrane (Nishikawa and Nakano, 1991). In vivo and in vitro analyses have indicated that the interaction between Sar1p and Sec12p is critical for their functions (Nakano and Muramatsu, 1989; Oka et al., 1991; d'Enfert et al., 1991*b*). 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., 1991*b*; Rexach and Schekman, 1991).

In this paper, we show that in our cell-free assays Sar1p-GTPγS 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 Sar1p. The functional roles of Sar1p 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 X2180-1A (*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 MAT*a) (Nakano et al., 1988), and MBY8-20C (*sec23-1 ura3-52 leu2-3, 112 trp1-289 his3 his4 gal2 MAT*α) (Hicke and Schekman, 1989). The cells were grown at 30 or 24°C (for *sec* mutants) in YPD medium (2% polypeptone [Nihon Pharmaceutical Co. Ltd., Tokyo, Japan], 1% yeast extract [Difco Laboratories, Inc., Detroit, MI] and 2% glucose).

In Vitro Transport Reaction

[^{35}S]-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]$_2$). At this point, ~20% of prepro-α-factor was translocated into the ER but virtually no transport to the Golgi was detected. Then the reaction was diluted fourfold with the reaction buffer and centrifuged at 8,000 *g* for 45 s. The washed semi-intact cells were reincubated at either 17 or 27°C for 60 min with the *sec23* cytosol and ATP-regeneration system. The reaction was stopped by the addition of an equal volume of 2% SDS, heated at 100°C for 5 min, and subjected to immunoprecipitation with anti-prepro-α-factor and anti-α1→6 mannosyl linkage antibodies (see Baker et al., 1988). The radioactive immunoprecipitates were dissolved in 2% SDS and analyzed by scintillation counting using solid scintillator Ready Cap (Beckman Instruments Inc., Palo Alto, CA). Recombinant Sar1p produced in *E. coli* was partially purified according to the method of Oka et al. (1991).

Vesicle Formation Assay

After a standard transport reaction was performed, the reaction mixture was centrifuged at 20,000 *g* for 1 min. The resultant supernatant (S-20) was treated with 0.47 mg/ml trypsin on ice for 30 min, and then with 1.53 mg/ml trypsin inhibitor. The mixture was heated at 100°C in the presence of 1% SDS and subjected to precipitation with the anti-prepro-α-factor antibody or with Con A-Sepharose. The radioactive precipitates were analyzed either by SDS-PAGE and fluorography or by scintillation counting.

Chase Reaction of Intermediate Vesicles to the Golgi

Semi-intact cells were incubated with Sar1p-GTPγS 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-α1→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 vesicle 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 63 h in Hitachi RPS65T rotor and fractionated. The distribution of vesicles was analyzed as above.

To demonstrate the presence of Sar1p on the vesicles, we employed glycerol density gradient centrifugation. The S-20 fraction from the reaction accumulating vesicles was centrifuged at 100,000 *g* for 60 min through a 25% (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), and 0.42 ml each of 30 and 25% (wt/vol) glycerol solution. 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 immunoblotting with the anti-Sar1p antiserum. The ER-form of pro-α-factor in each fraction was analyzed by immunoprecipitation with the anti-prepro-α-factor antibody or measured in the blot by Imageanalyzer bass-2000 (Fuji Film 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 Sar1p-GTPγS

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 Sar1p. To further specify the steps requiring the functions of Sec12p and Sar1p, 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 Sar1p. 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 Sar1p, confirming the observation of Rexach and Schekman (1991) that the *sec12* membrane is defective in the formation of vesicles. Fig. 1 *C*

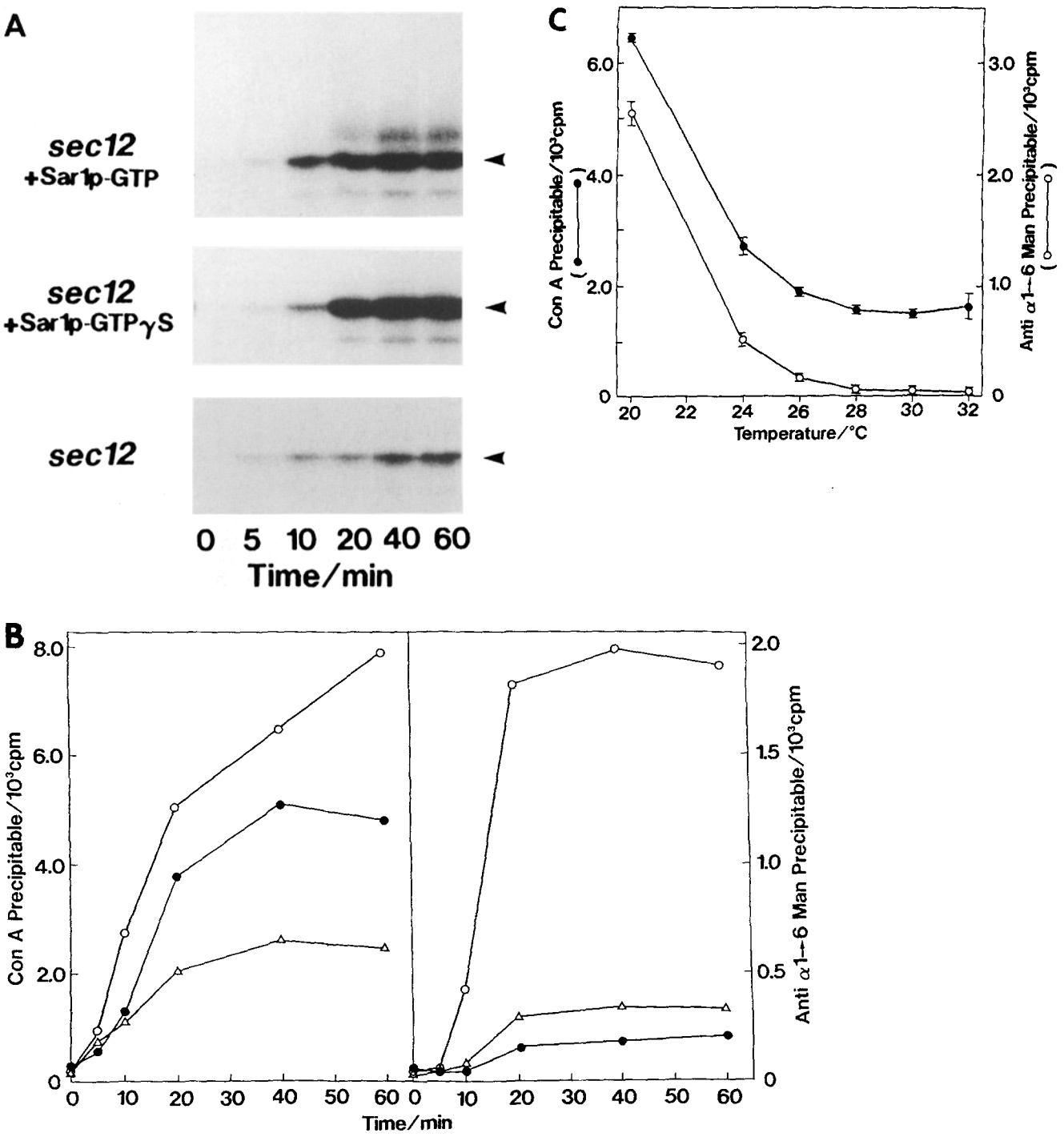

Figure 1. Sar1p-GTPγS suppresses the *sec12* defect in vesicle formation but blocks the transport to the Golgi apparatus. (*A*) The *sec12* semi-intact cells were incubated at 26°C in a standard cell-free transport reaction with 2 μg Sar1p-GTP, with 2 μg Sar1p-GTPγS, or without any additional Sar1p. 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*). (○) *sec12* membranes plus Sar1p-GTP, (●) *sec12* membranes plus Sar1p-GTPγS, (△) *sec12* membranes without Sar1p. (*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 (○).

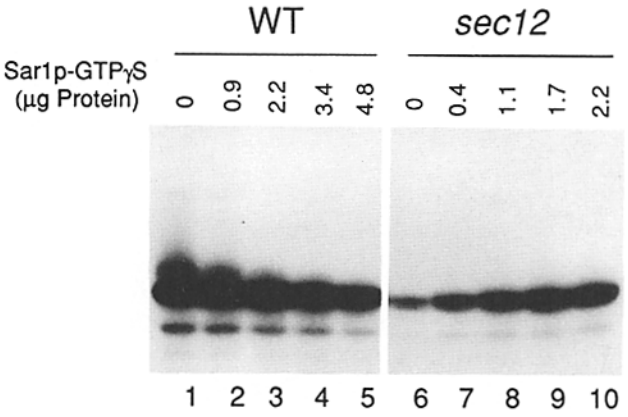

Figure 2. Sar1p-GTPγS accumulates vesicles in a dose-dependent manner. The wild-type (*WT*) and *sec12* semi-intact cells were incubated for 60 min with the indicated amount of Sar1p-GTPγS at 20 and 26°C, respectively. After reaction, S-20 fraction was prepared as in Fig. 1 *A* and analyzed by immunoprecipitation with anti-prepro-α-factor antibody.

shows the temperature dependence of vesicle formation by the *sec12* semi-intact cells (shown as Con A precipitable counts; *closed circles*). The decrease of the Con A-precipitable species was drastic upon shift from 20 to 26°C. This profile is quite similar to that of the overall ER-to-Golgi transport (measured by acquisition of α1→6 mannosyl linkage; *open circles*) (see also Oka et al., 1991), implying that the primary defect of *sec12* is in the formation of vesicles.

The addition of Sar1p-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*. Sar1p-GTP restored the ability of the *sec12* membrane to form vesicles (Con A-precipitable pro-α-factor) at 26°C to the level of the permissive temperature (compare with Fig. 1 *C*). Furthermore, the incubation with Sar1p-GTP gave rise to the Golgi-form of pro-α-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 Sar1p-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 Sar1p-GTPγS (Fig. 1 *A*, *middle panel*), the ER-form of pro-α-factor was liberated into the vesicle fraction as efficiently as in the case of Sar1p-GTP. However, Sar1p-GTPγS 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 Sar1p 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

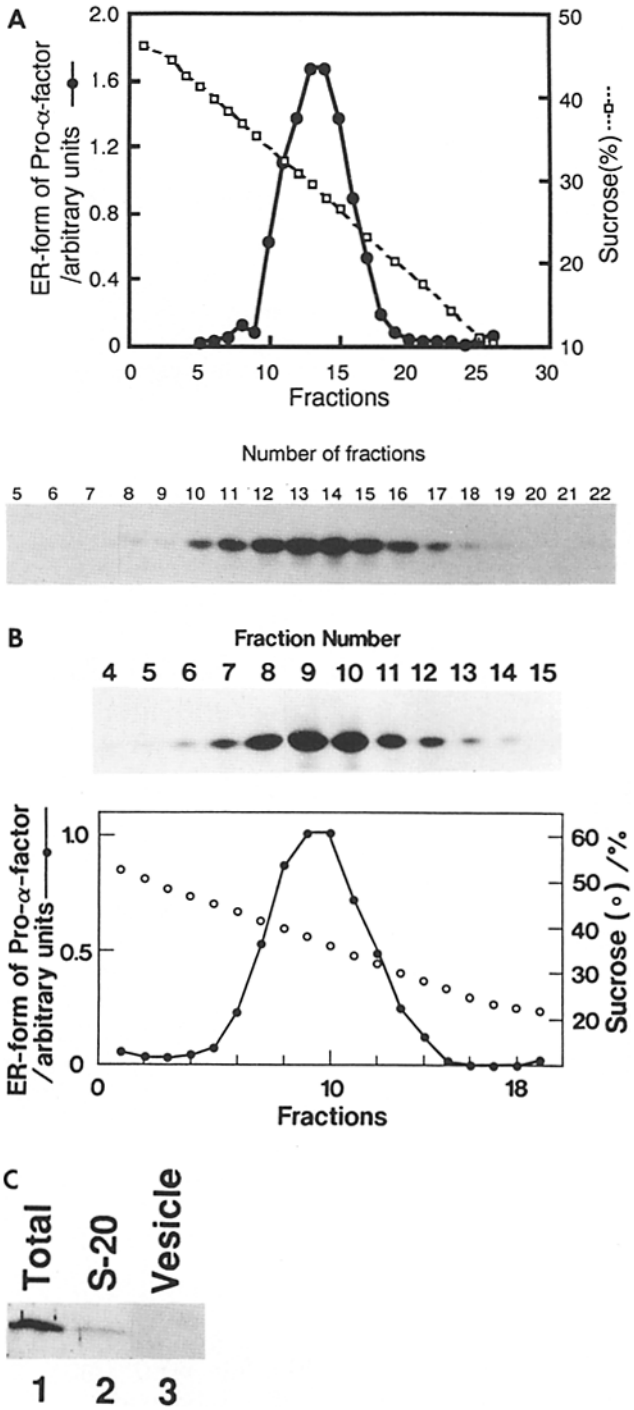

Figure 3. Analysis of vesicles accumulated by Sar1p-GTPγS by sucrose density gradient centrifugation. (*A*) S-20 fraction was prepared from the incubation of the *sec12* semi-intact cells with Sar1p-GTPγS at 27°C for 60 min and centrifuged at 100,000 *g* for 60 min through a 15% (wt/vol) sucrose cushion. The pellet was resuspended in 10% sucrose and placed on the top of a 15–45% linear sucrose density gradient. After ultracentrifugation at 120,000 *g* for 140 min, fractions were collected from the bottom. The density of sucrose of each fraction was measured using a refractometer (□). The ER-form of pro-α-factor was analyzed by immunoprecipitation with anti-prepro-α-factor antibody and fluorography (●). (*B*) The peak fractions of the velocity sucrose gradient (*A*) were analyzed by an equilibrium flotation through a 20–50% linear sucrose gradient (120,000 *g*, 63 h). The ER-form of pro-α-factor was analyzed by immunoprecipitation (●). (*C*) The S-20 fraction containing the accumulated vesicles was subjected to a sucrose density gradient centrifugation as described in *A*. The total reaction mixture after the transport reaction (*Total*), the S-20 fraction (*S-20*), and the peak fractions of the sedimentation centrifugation (*Vesicle*) were analyzed by immunoblotting using the anti-Sar1p antibody. The amount of fractions run in the gel corresponded to two standard reactions for *Total* and *S-20* and nine reactions for *Vesicle*.

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 *secl2* 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 *secl2* 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 GTPγS seemed to be a dead end product of the reaction and were not able to proceed to further steps of transport (Melançon 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 ATP and cytosol, and incubated with fresh wild-type semi-intact cells. Approximately 15–20% of the ER-form in the vesicles was converted to the Golgi-form. As shown in Fig. 4 *A*, this chase reaction was dependent on ATP and the added semi-intact cells. The antibody against Yptlp, another GTPase which functions in the late steps of transport, inhibited the reaction. This inhibition by anti-Yptlp antibody was canceled by purified Yptlp. Additional Sarlp did not affect the reaction irrespective of GTP- or GTPγS-bound form. Thus the transport to the Golgi in this chase reaction requires Yptlp but presumably not Sarlp.

Since the vesicle fraction we used contained a small amount of prepro-α-factor (data not shown), it is possible that this precursor gets translocated into the ER of the fresh semi-intact cells and further transported to the Golgi during

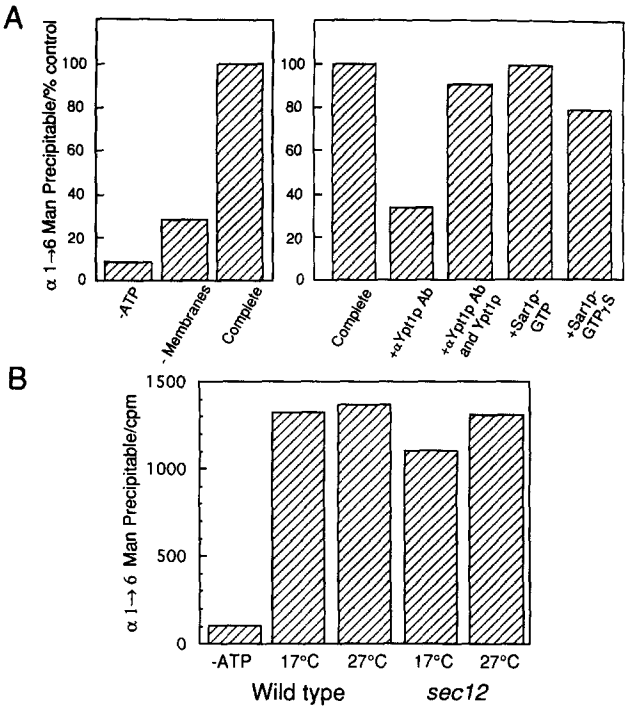

Figure 4. Vesicles accumulated by Sarlp-GTPγS are a functional intermediate of the ER-to-Golgi transport. (*A*) The wild-type semi-intact cells were incubated with Sarlp-GTPγS at 20°C for 60 min. The S-20 fraction was prepared and centrifuged at 100,000 *g* for 30 min through 1 M sorbitol cushion. The 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 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 *secl2* 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 *secl2* 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 Sec12p is not required in the later steps of transport.

These results have led us to conclude that the vesicles accumulated by Sar1p-GTPγS 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 Sar1p and the late one dependent on Ypt1p.

Sar1p Is Present on the Intermediate Vesicles

All the results hitherto described suggest that Sar1p has to fulfill its GTPase activity after vesicles are completed and released from the ER. Since the GTP hydrolysis by Sar1p is essential for entering subsequent reactions of vesicular targeting to the Golgi, it would be most reasonable that Sar1p functions on the vesicles. However, despite strenuous efforts we could not prove the presence of Sar1p in the vesicle fractions of the sucrose density gradient. Fig. 3 *C* shows one of such analyses; Sar1p 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 Sar1p 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 Sar1p-GTPγS, this peak was not observed at all (Fig. 5 *C*, *open circles*). The glycerol gradient fractions generated by the incubation with Sar1p-GTPγS were analyzed by immunoblotting using the anti-Sar1p antibody. As shown in Fig. 5 *A* (also in *closed circles* in Fig. 5 *B*), Sar1p clearly comigrated with the peak of pro-α-factor, indicating that Sar1p is present on the vesicles. This supports the view that Sar1p executes its GTPase activity on the vesicles.

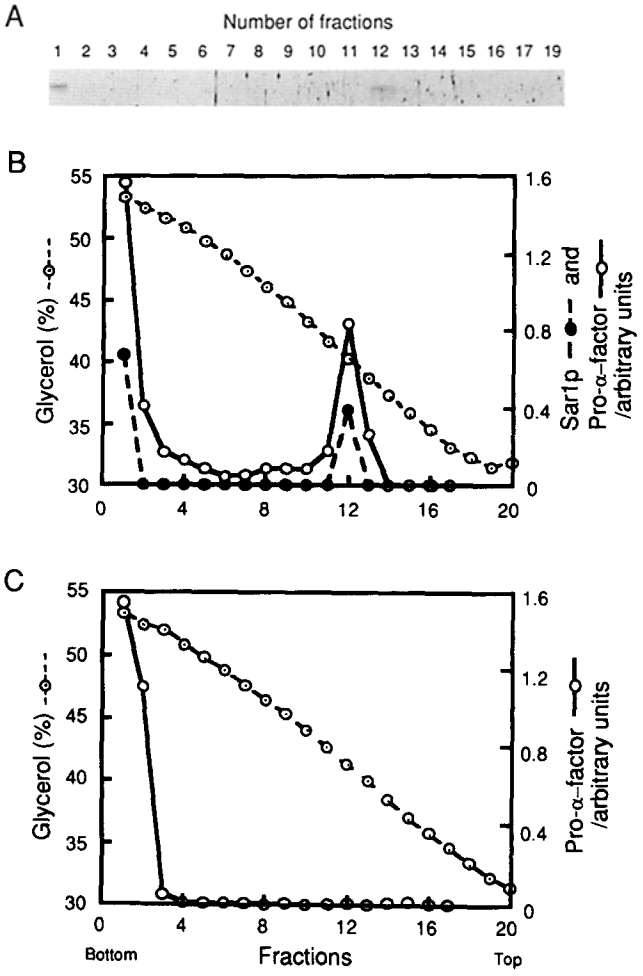

Figure 5. Sar1p comigrates with the vesicles in a glycerol density gradient. The *sec12* semi-intact cells were incubated with (*A* and *B*) or without Sar1p-GTPγS (*C*) at 27°C for 60 min. S-20 fraction was prepared and centrifuged at 100,000 *g* for 60 min through a 25% (wt/vol) glycerol solution. The pellet was resuspended in a 55% glycerol solution, placed at the bottom of a tube and overlaid with 50–25% (wt/vol) glycerol solutions. The gradient was centrifuged (120,000 *g* for 46.5 h) and fractionated. The density of glycerol was determined by refractometry (⊙). Each fraction was subjected to SDS-PAGE and immunoblotting for the analysis of the ER-form of pro-α-factor (○) and Sar1p (●). For details see under Materials and Methods.

Sar1p-GTPγS 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 Sar1p, 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 Sar1p 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 Sar1p, perhaps between the vesicles and the ER membrane, and that this may eventually lead to the shortage of active Sar1p 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*), Sar1p-GTPγS promoted the release of vesicles but it plateaued at a lower level than the case of Sar1p-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 Sar1p (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

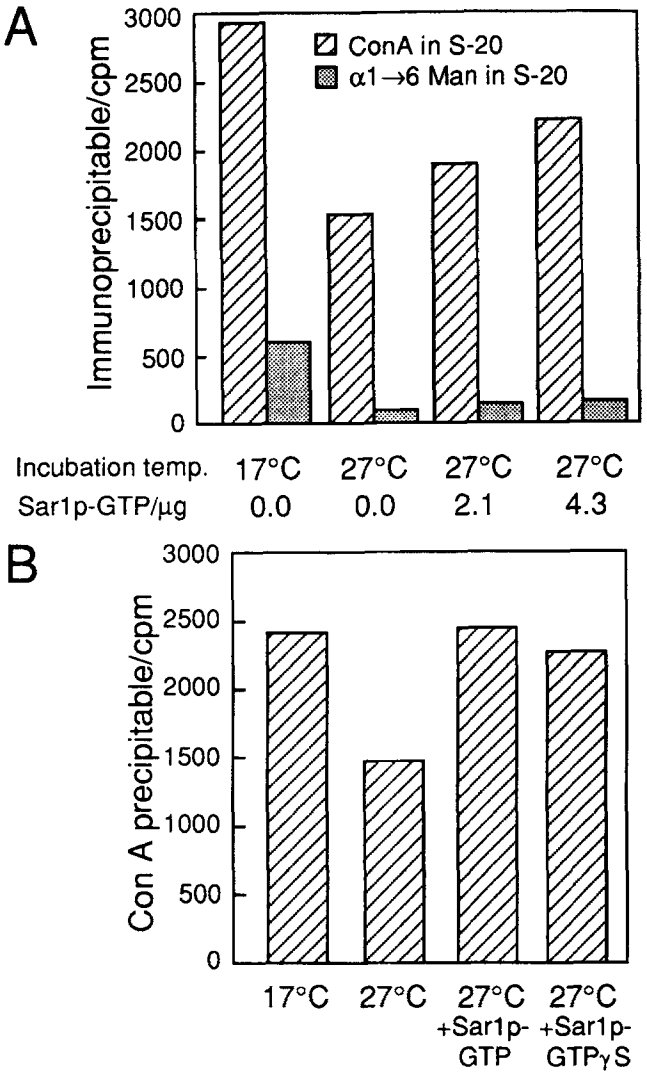

Figure 6. Sarlp suppresses the *sec23* defect in vesicle formation. (*A*) The *sec23* semi-intact cells were incubated with the indicated amounts of Sarlp-GTP at 17 or 27°C. To avoid the effect of the wild-type Sec23 protein present in the prepro-α-factor preparation, a two-step reaction procedure was 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*).

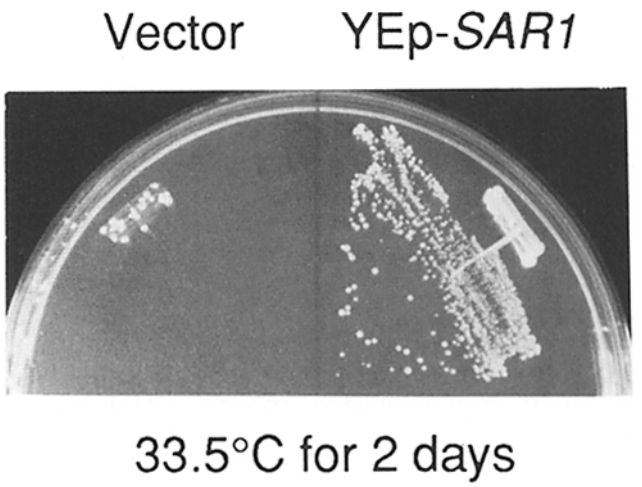

Figure 7. Overexpression of *SAR1* partially suppresses the temperature-sensitive growth of the *sec23* mutant. The *sec23-1* mutant cells (MBY8-20C) harboring *SAR1* on a multicopy plasmid (pANY2-7) or the vector alone (pSEY8) (see Nakano and Muramatsu, 1989) were streaked on a YPD plate and incubated for 2 d at 33.5°C, a semi-restrictive temperature for *sec23-1*.

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 *SAR1* 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

and Schekman, 1991; this study). 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 Sar1p (Barlowe and Schekman, 1993). The rescue of the *sec12* defect by Sar1p is thus easily explained by the supplementation of the active form (i.e., GTP-bound form) of Sar1p.

We previously reported that Sar1p-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 Sar1p. 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 Sar1p in either the GTP- or GTPγS-bound form. Sar1p-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 Sar1p-GTPγS added, indicating that the active form of Sar1p is limiting in the *sec12* membranes. It should be pointed out here that Sar1p-GTP is more active than Sar1p-GTPγS in the vesicle formation. This is probably because the hydrolysis of GTP enables the cycling of GTP- and GDP-bound states of Sar1p and thus drives turnover of the budding reaction. In fact, the vesicle production by Sar1p-GTP continues to increase whereas that by Sar1p-GTPγS plateaus in time course experiments (see Fig. 1 *B*).

In contrast to the vesicle formation, Sar1p-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 Sar1p-GTPγS accumulate in the supernatant. Sar1p-GTPγS also shows a transdominant effect to the wild-type membranes. The step of blockade 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 Sar1p (Yoshihisa 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 Sar1p 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 Sar1p in a dose-dependent manner. In this suppression, Sar1p-GTPγS is as active as Sar1p-GTP, supporting our conclusion that the GTP hydrolysis by Sar1p 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 Sar1p cycling between the vesicles and the ER. Presumably the GTPase activity is required for the release of Sar1p from the vesicles. The lesion in the GAP activity of Sec23p would then block the return of Sar1p to the ER, which eventually leads to the deficiency of the active form of Sar1p 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 Sar1p-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 Ypt1p inhibits this chase reaction. Thus the vesicles accumulated by Sar1p-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 Sar1p-GTPγS. This suggests that functions of Sec12p and Sar1p are only required in the early events but not in the later targeting reactions.

Function of Sar1p as a GTPase

We were able to demonstrate that Sar1p itself is present on the vesicles accumulated by Sar1p-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 Sar1p and instead regain the targeting competence. These findings suggest that Sar1p fulfills its GTPase activity on the completed vesicles and that the release of Sar1p-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 Sar1p-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 Sar1p-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 Sar1p-GTPγS represent a functional intermediate of the ER-to-Golgi transport.

Based on the data available, we propose a model of Sar1p 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 Sar1p, which then converts Sar1p from the inactive GDP-form to the active GTP-form. Sar1p-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 coatomers [Hosobuchi et al., 1992]). Then Sec23p acts on Sar1p to activate GTPase. The block of GTP hydroly-

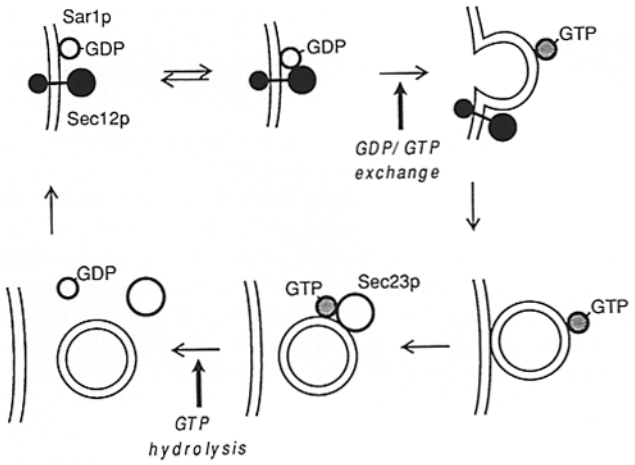

Figure 8. Models for the Sar1p function in the early events of vesicle formation from the ER. See text for discussion.

sis by either GTPγS or the *sec23* mutation would keep Sar1p sitting on the vesicle, which prevents entering the next step of transport. If Sar1p 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 Sar1p-GTPγS does not inhibit the later reaction may indicate that the release of Sar1p provokes a substantial change in the properties of the vesicles. In this regard, it may be worth mentioning that the vesicles yielded by Sar1p-GTPγS are slightly but reproducibly denser than the vesicles accumulated by the action of the anti-Ypt1p 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 Sar1p promotes budding and completion of a vesicle but the block of GTP hydrolysis by GTPγS 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 Sar1p. 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., 1991*a*; 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 Sar1p is present on our vesicle fraction but is not highly enriched. Presumably only a catalytic amount of Sar1p is enough to execute its function on the vesicle. Alternatively, the vesicles we prepared might have lost a large part of the bound Sar1p as discussed above, even though they were isolated by glycerol gradient. It would be also interesting to examine whether other membrane proteins such as Bos1p (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, Sar1p and Ypt1p. 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 Ypt1p is already present in the vesicle fraction produced by Sar1p-GTPγS (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 Sar1p as a GTPase, genetic approach should also be very useful. We have recently obtained 3 temperature-sensitive and 7 dominant-negative alleles of *SAR1* by random and site-directed mutagenesis (Nakano, A., H. 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 *SAR1* 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.

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References

Baker, D., L. Hicke, M. Rexach, M. Schleyer, and R. Schekman. 1988. Reconstitution of *SEC* gene product-dependent intercompartmental protein transport. *Cell.* 54:335–344.

Balch, W. E. 1990. Small GTP-binding proteins in vesicular transport. *Trends Cell Biol.* 15:473–477.

Barlow, C., and R. Schekman. 1993. *SEC12* encodes a guanine nucleotide exchange factor essential for transport vesicle budding from the ER. *Nature (Lond.).* 365:347–349.

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

d'Enfert, C., C. Barlowe, S. Nishikawa, A. Nakano, and R. Schekman. 1991*a*. Structural and functional dissection of a membrane glycoprotein required for vesicle budding from the endoplasmic reticulum. *Mol. Cell. Biol.* 11:5727–5734.

d'Enfert, C., L. J. Wuestehube, T. Lila, and R. Schekman. 1991*b*. Sec12p-dependent membrane binding of the small GTP-binding protein Sar1p promotes formation of transport vesicles from the ER. *J. Cell Biol.* 114:663–670.

Franzusoff, A., E. Lauzé, and K. E. Howell. 1992 Immuno-isolation of Sec7p-coated transport vesicles from the yeast secretory pathway. *Nature (Lond.).* 355:173–175.

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

Groesch, M. E., H. Ruohola, R. Bacon, G. Rossi, and S. Ferro-Novick. 1990. Isolation of a functional vesicular intermediate that mediates ER to Golgi transport in yeast. *J. Cell Biol.* 111:45–53.

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

Hosobuchi, M., T. Kreis, and R. Schekman. 1992. *SEC21* is a gene required for ER to Golgi protein transport that encodes a subunit of a yeast coatomer. *Nature (Lond.).* 360:603–605.

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

Lian, J. P., and S. Ferro-Novick. 1993. Bos1p, an integral membrane protein of the endoplasmic reticulum to Golgi transport vesicles, is required for their fusion competence. *Cell.* 73:735–745.

Lodish, H. F., N. Kong, S. Hirani, and J. Rasmussen. 1987. A vesicular intermediate in the transport of hepatoma secretory proteins from the rough endoplasmic reticulum to the Golgi complex. *J. Cell Biol.* 104:221–230.

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

Nakano, A., and M. Muramatsu. 1989. A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Biol.* 109:2677–2691.

Nakano, A., D. Brada, and R. Schekman. 1988. A membrane glycoprotein, Sec12p, required for protein transport from the endoplasmic reticulum to the

Golgi apparatus in yeast. *J. Cell Biol.* 107:851–863.
Newman, A. P., J. Graf, P. Mancini, G. Rossi, J. P. Lian, and S. Ferro-Novick. 1992. *SEC22* and *SLY2* are identical. *Mol. Cell. Biol.* 12:3663–3664.
Nishikawa, S., and A. Nakano. 1991. The GTP-binding Sar1 protein is localized to the early compartment of the yeast secretory pathway. *Biochim. Biophys. Acta.* 1093:135–143.
Nishikawa, S., and A. Nakano. 1993. Identification of a gene required for membrane protein retention in the early secretory pathway. *Proc. Natl. Acad. Sci. USA.* 90:8179–8183.
Oka, T., S. Nishikawa, and A. Nakano. 1991. Reconstitution of GTP-binding Sar1 protein function in ER to Golgi transport. *J. Cell Biol.* 114:671–679.
Ossig, R., C. Dascher, H. H. Trepte, H. D. Schmitt, and D. Gallwitz. 1991. The yeast *SLY* gene products, suppressors of defects in the essential GTP-binding Ypt1 protein, may act in endoplasmic reticulum-to-Golgi transport. *Mol. Cell. Biol.* 11:2980–2993.
Palade, G. 1975. Intracellular aspects of the process of the protein secretion. *Science (Wash. DC).* 189:347–358.
Paulik, M., D. D. Nowack, and D. J. Morre. 1988. Isolation of a vesicular intermediate in the cell-free transfer of membrane from transitional elements of the endoplasmic reticulum to Golgi apparatus cisternae of rat liver. *J. Biol. Chem.* 263:17738–17748.
Pfeffer, S. R. 1992. GTP-binding proteins in intracellular transport. *Trends Cell Biol.* 2:41–46.
Pryer, N. K., N. R. Salama, R. Schekman, and C. A. Kaiser. 1993. Cytosolic Sec13p complex is required for vesicle formation from the endoplasmic reticulum in vitro. *J. Cell Biol.* 120:865–875.
Rexach, M. F., and R. W. Schekman. 1991. Distinct biochemical requirements for the budding, targeting, and fusion of ER-derived transport vesicles. *J. Cell Biol.* 114:219–229.
Ruohola, H., A. K. Kabcenell, and S. Ferro-Novick. 1988. Reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex in yeast: the acceptor Golgi compartment is defective in the *sec23* mutant. *J. Cell Biol.* 107:1465–1476.
Salminen, A., and P. J. Novick. 1987. A *ras*-like protein is required for a post-Golgi event in yeast secretion. *Cell.* 49:527–538.
Schmitt, H. D., M. Puzicha, and D. Gallwitz. 1988. Study of a temperature-sensitive mutant of the *ras*-related *YPT1* gene product in yeast suggests a role in the regulation of intracellular calcium. *Cell.* 53:635–647.
Segev, N. 1991. Mediation of the attachment or fusion step in vesicular transport by the GTP-binding Ypt1 protein. *Science (Wash. DC).* 252:1553–1556.
Segev, N., J. Mulholland, and D. Botstein. 1988. The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. *Cell.* 52:915–924.
Yoshihisa, T., C. Barlowe, and R. Schekman. 1993. Requirement for a GTPase-activating protein in vesicle budding from the endoplasmic reticulum. *Science (Wash. DC).* 259:1466–1468.