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# Activities of Mutant Sar1 Proteins in Guanine Nucleotide Binding, GTP Hydrolysis, and Cell-Free Transport from the Endoplasmic Reticulum to the Golgi Apparatus[1]

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**Sar1p belongs to a unique subfamily of the small GTPase superfamily and is essential for the formation of vesicles that transport proteins from the endoplasmic reticulum to the Golgi apparatus. We have obtained mutants of the yeast *SAR1* gene, which show several different phenotypes in cell growth and protein transport [Nakano, A., Otsuka, H., Yamagishi, M., Yamamoto, E., Kimura, K., Nishikawa, S., and Oka, T. (1994) *J. Biochem.* 116, 243-247; Yamanushi, T., Hirata, A., Oka, T., and Nakano, A. (1996) *ibid.* 120, 452-458]. In this study, we have purified five mutant Sar1 proteins using an *Escherichia coli* expression system and characterized their biochemical properties in detail. Three of them prefer GDP binding to GTP binding and are thus regarded as GDP-form mutants, and one is insensitive to the GTPase-activating protein and is almost fixed in the GTP-bound state. The GDP mutants are defective in vesicle formation *in vitro*, whereas the GTP mutant can drive vesicle formation but not the overall transport to the Golgi. These mutants will be useful for further understanding of the regulation of the GTPase cycle of Sar1p.**

**Key words: COPII vesicle formation, *SAR1*, small GTPase, vesicular transport, yeast *Saccharomyces cerevisiae*.**

GTPases are known to regulate a variety of cellular reactions by acting as molecular switches (*1, 2*). A huge number of low-molecular-weight GTPases have been discovered and are now collectively called the Ras-superfamily. One of the very important findings in the last decade is that many small GTPases are involved in intracellular protein trafficking. The Rab/Ypt and Sar/Arf families are required for the regulation of vesicular transport (*3, 4*), and the Ran family is important for nuclear import and export (*5*).

Many biochemical data so far accumulated indicate that the two families involved in vesicular traffic, Rab/Ypt and Sar/Arf, play different roles in each step of transport. In the case of the transport from the endoplasmic reticulum (ER) to the Golgi apparatus in yeast, for example, Sar1p is essential for the budding and formation of transport vesicles from the ER (*6-8*), whereas Ypt1p is needed for the targeting and fusion of the vesicles to the Golgi membrane (*9, 10*).

From the structural point of view, the Sar/Arf family of proteins are the most distant relatives in the Ras-superfamily. Phylogenetic analysis predicts that this family forms a quite isolated branch from other members of the superfamily (*11, 12*). Furthermore, the mode of posttranslational modification is totally different. Most other families of small GTPases, including Ras, Rho/Rac, and Rab/Ypt, have cysteine residue(s) at their C-termini, which are modified with lipidic moieties, farnesyl or geranylgeranyl (*13*). The Sar and Arf proteins do not have such prenyl modifications. Instead, Arf proteins commonly have a myristoyl modification at their N-termini, which is essential for their activity (*14, 15*). Sar1p is quite unique in this regard; it does not contain the sequence to be modified with myristic acid either (*7*). Sar1p has no posttranslational modification at all and is purely a simple polypeptide.

We have been studying the role of the GTPase cycle of yeast Sar1p in terms of the regulation of vesicle budding and protein sorting. To take advantage of the yeast genetic system, we have constructed eight mutant versions of Sar1p (D32G, K36M, T54A, D73V, H77L, E112K, N132I, and C171S). Numerous mutational studies have been performed on Ras proteins, and now it is widely believed that particular types of mutations cause alteration of GDP/GTP binding and GTPase activities in common (*11, 12*). In fact, most of the mutations we introduced into Sar1p were designed based on the knowledge on Ras (see Table III). However, great care should be taken if one wants to study

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Abbreviations: ER, endoplasmic reticulum; GAP, GTPase-activating protein; GEF, guanine-nucleotide exchange factor.

a unique subfamily of proteins. There is no assurance that a similar mutation gives a similar outcome if the proteins are quite diverged. In the case of the Sar/Arf family, the work on yeast Arf1p by Kahn *et al.* (*16*) is the only biochemical analysis made so far on a purified protein. To address this problem, we purified the wild-type and five mutant versions of Sar1p, and performed detailed characterization of them: guanine nucleotide binding, GTPase activity, and the ability to promote ER-to-Golgi transport in a cell-free reaction. The usability of the mutations we constructed will be discussed.

## MATERIALS AND METHODS

*Strains*—The yeast *Saccharomyces cerevisiae* strains used in this study were X2180-1A (*MAT***a** *mal gal2 CUP1*) (Yeast Genetic Stock Center, Berkeley, CA), MBY10-7A (*MAT***a** *sec12-4 ura3-52 leu2-3, 112 trp1-289 his3 his4 suc gal2*) (*17*), and RSY620 (*MAT***a** *ura3-52 leu2-3, 112 ade2-1 trp1-1 his3-11, 15 can1-100 pep4::TRP1*/ pTYY117 [*SEC24*-$HIS_6$ inserted into YEp352]/pTYY121 [*SEC23* inserted into YEp351]) (*18*). The *Escherichia coli* strain used for Sar1p purification was BL21 (DE3) [$F^-$ *ompT* $r_B^- m_B^-$]/pLysS (*19*)/pMYE3-1 (*20*).

*Purification of Sar1 Proteins*—Purification of the wild-type and mutant Sar1p using an *E. coli* expression system was performed as described (*20*). For purification of the Sar1 E112K mutant protein (Sar1pE112K), the induction conditions were slightly modified. *E. coli* BL21 (DE3)/ pLysS cells carrying the Sar1pE112K expression plasmid (pMYE3-9) were cultured at 30°C in 1 liter LB medium containing 50 $\mu$g/ml ampicillin and 25 $\mu$g/ml chloramphenicol to an early log phase ($OD_{650}$ 0.25). Synthesis of Sar1p was induced by the addition of 1 mM isopropylthiogalactoside, and then incubation was continued for an additional 3 h. Cells were harvested at a middle log phase ($OD_{650}$ 0.55). All subsequent procedures, including cell lysis by sonication, DEAE-Sephacel column chromatography, Sephacryl S-200 gel filtration and high-performance liquid chromatography on a DEAE-5PW column, were performed exactly as described (*20*).

*Purification of the Sec23p-Sec24p Complex*—Purification of the Sec23p-Sec24p complex was performed as previously described (*18*) with some modifications. Yeast strain RSY620 was grown in 1.2 liters of MVD medium (0.67% yeast nitrogen base, 2% glucose) containing 0.1 M potassium phosphate buffer (pH 6.5) and appropriate supplements to a late log growth phase ($OD_{600}$ 0.4), and then harvested and washed with distilled water. All subsequent steps were performed at 4°C. Cells (20 g) were resuspended in 20 ml buffer A [50 mM HEPES-KOH, pH 6.8, 50 mM potassium acetate (KOAc), 5 mM EDTA, 0.5 mM PMSF] and then disrupted in a bead-beater chamber with a 1/2 volume of glass beads. The crude homogenate was centrifuged at 30,000 × *g* for 10 min and the supernatant was set aside. The pellet was resuspended in 60 ml buffer A containing 0.75 M KOAc, and the lysis procedure was repeated to extract additional Sec23p complex. The two supernatants were combined and centrifuged at 100,000 × *g* for 90 min. Solid $(NH_4)_2SO_4$ was dissolved in the lysate to 30% saturation, and the resulting precipitate was collected by centrifugation at 30,000 × *g* for 15 min. The pellet was resuspended in 10 ml of 50 mM HEPES-KOH, pH 6.8, and 0.2 mM EGTA. The suspension was further made to 0.5 M KOAc and subjected to HPLC on a TSK DEAE-5PW semi-prep column (21.5 mm × 15 cm, Tosoh), which had been equilibrated with 50 mM HEPES-KOH, pH 6.8, 0.25 M sorbitol, 0.2 mM EGTA, and 0.5 M KOAc. The Sec23p complex was eluted with a linear gradient of 0.5-0.8 M KOAc at the flow rate of 4.5 ml/min. The pure Sec23p complex peak fractions were pooled based on the results of analysis by immunoblotting with an anti-Sec23p antibody and Coomassie Brilliant Blue staining.

*Guanine-Nucleotide-Binding Assays*—GTP$\gamma$S- and GDP-binding activities were measured by the procedure described by Kimura *et al.* (*20*). Sar1p (0.15 $\mu$M) was incubated with 0.1-2.0 $\mu$M [$^{35}$S]GTP$\gamma$S or [$^3$H]GDP (10,000 dpm/pmol, DuPont New England Nuclear) in 40 $\mu$l reaction buffer (0.075% Triton X-100, 25 mM HEPES-KOH, pH 6.8, 1 mM $MgCl_2$, and 0.25 mg/ml bovine serum albumin) at 20 or 30°C. The reaction was stopped by the addition of 800 $\mu$l chilled stop buffer (0.002% Triton X-100, 25 mM HEPES-KOH, pH 6.8, 100 mM NaCl, and 5 mM $MgCl_2$), and then immediately filtrated through a nitrocellulose filter (Millipore HA). The filter was quickly washed four times with 2 ml of stop buffer, and then dried and subjected to measurement of the radioactivity by scintillation counting.

*GTPase and GAP Assays*—The ability of Sar1p to hydrolyze GTP was measured by quantifying GTP and GDP separated on thin-layer plates (*7*). Purified Sar1p (0.1 pmol/$\mu$l of GTP$\gamma$S-binding site) was incubated in 20 $\mu$l volume with 0.2 $\mu$M [$\alpha$-$^{32}$P] GTP (20,000 dpm/pmol, DuPont New England Nuclear), 0.1% Triton X-100, 25 mM HEPES-KOH, pH 6.8, 1 mM magnesium acetate, 0.5 mM dithiothreitol, and 0.25 mg/ml bovine serum albumin at 30°C. The reactions were stopped by the addition of 2 $\mu$l of 200 mM EDTA, followed by placing on ice. A 1 $\mu$l aliquot of the reaction mixture was spotted onto a polyethyleneimine cellulose plate (Sigma) and developed with 1 M LiCl/1 M HCOOH (1 : 1, v/v). The plate was dried, autoradiographed on an imaging plate (BAS-SR 2040, Fuji Photo Film) and quantitatively analyzed with a BIO-imaging Analyzer (BAS 2500, Fuji Photo Film). For the GAP assay, 0.1 pmol/$\mu$l of Sec23p-Sec24p complex was added to the reaction.

*Cell-Free Transport Reaction*—Transport-competent semi-intact cells and cytosol were prepared as described (*21*). The strains MBY10-7A and X2180-1A were used to prepare the *sec12* semi-intact cells and the wild-type cytosol, respectively. The transport marker, $^{35}$S-labeled prepro-$\alpha$-factor, was prepared by *in vitro* transcription and translation by the procedure of Oka *et al.* (*22*). Standard transport reaction and vesicle formation assay were performed as described (*8, 22*).

## RESULTS

*Purification of Sar1p*—We have established a purification procedure for the wild-type Sar1p, based on an *E. coli* expression system (*20*). From the supernatant of a sonication lysate of *E. coli*, Sar1p can be purified to homogeneity by three-step protocol: anion-exchange column chromatography, gel filtration, and HPLC. This procedure has the advantage for purifying mutant Sar1p over the method based on overexpression in yeast (*7*), because most of the

mutant Sar1p we constructed cause lethality when overproduced in yeast cells (*23, 24*). By applying the same procedure, we could purify D32G, T54A, H77L, and C171S mutant versions of Sar1p. For the E112K mutant, we had to slightly optimize the induction conditions as described under "MATERIALS AND METHODS." Unfortunately, K36M, D73V, and N132I mutants behaved quite differently from other versions and we were unable to purify them by this protocol.

*Guanine Nucleotide Binding*—The purified Sar1 proteins were subjected to analysis of the activity of guanine nucleotide binding. Aliquots of Sar1p were mixed with [$^{35}$S]GTP$\gamma$S or [$^{3}$H]GDP and incubated at 20 or 30°C. The resultant complex was trapped on a nitrocellulose filter and washed, and then the guanine nucleotide bound to Sar1p was quantified by scintillation counting. Figure 1 shows the time course of the GTP$\gamma$S- and GDP-binding to the wild-type and mutant Sar1p. In the case of wild-type Sar1p (WT), the binding of either GTP$\gamma$S or GDP saturated during the incubation for 50 min at 20°C and 20 min at 30°C. For D32G and T54A mutants, however, GTP$\gamma$S binding was much lower than GDP-binding. Even on 60-min incubation, 10% or less GTP$\gamma$S could bind to Sar1p. The binding of GDP was normal for both of these mutants. It should be noted that the low GTP$\gamma$S-binding activity of D32G was even lower at 30°C. This is consistent with the

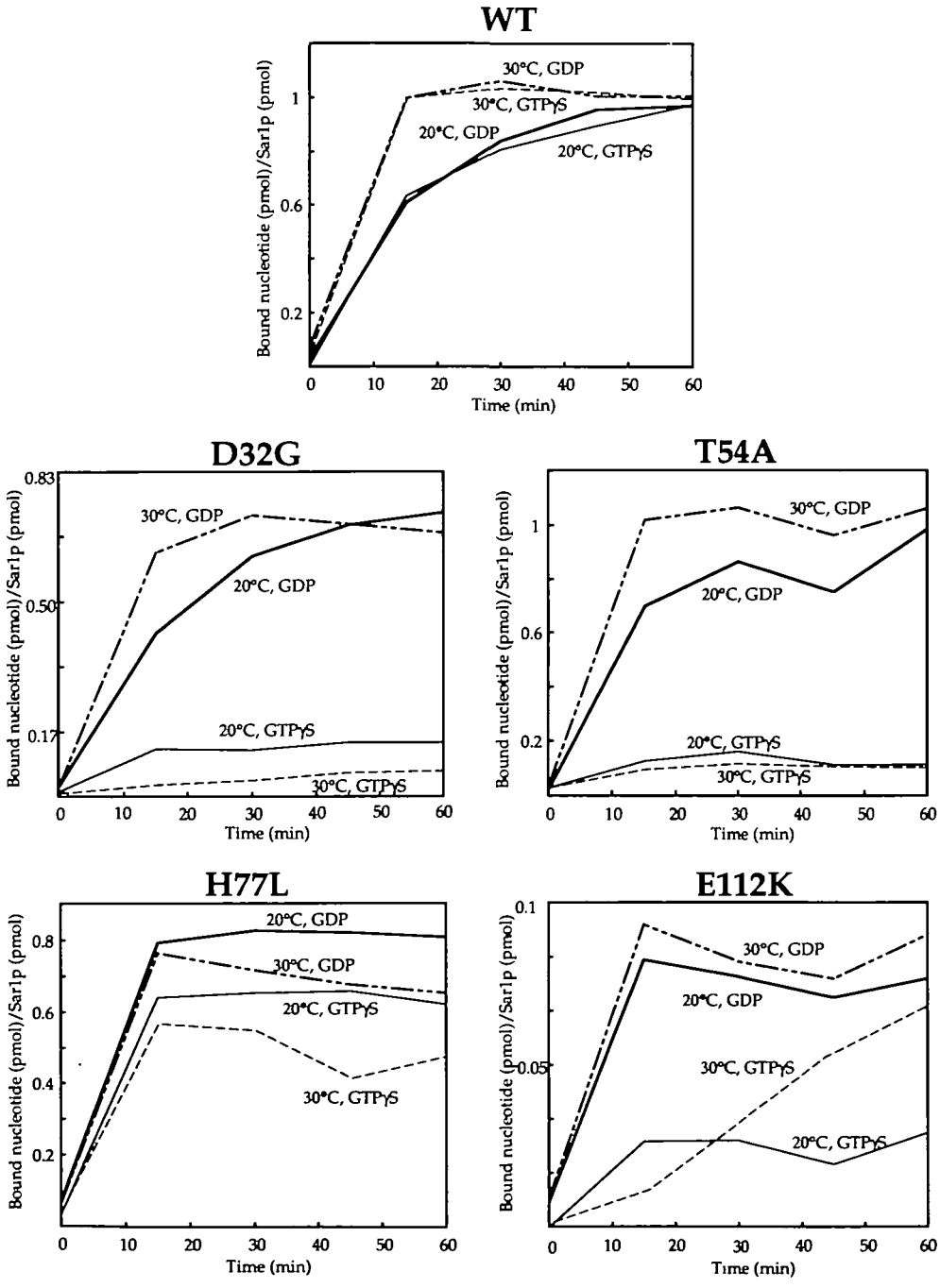


Fig. 1. **Time courses of GTP$\gamma$S- and GDP-binding.** Purified Sar1p (0.15 $\mu$M) was mixed with 2 $\mu$M [$^{35}$S]GTP$\gamma$S or [$^{3}$H]GDP (10,000 dpm/pmol) at the indicated temperatures. At appropriate time intervals, aliquots of the reaction mixture were filtrated through a nitrocellulose membrane filter and then the radioactivity of the bound nucleotide was measured by scintillation counting. Thin lines show [$^{35}$S]GTP$\gamma$S binding and thick lines [$^{3}$H]GDP binding. Solid and broken lines indicate the reactions at 20 and 30°C, respectively.

fact that the yeast cells that depend solely on D32G are temperature sensitive for growth (*23, 24*). The H77L mutant gave a similar profile to the wild type. The saturation level of GTP$\gamma$S-binding appeared to be a little lower than that of the wild-type Sar1p, but the time course itself showed a rapid profile. C171S also showed a normal profile almost indistinguishable from that of the wild type (data not shown).

The E112K mutant, in contrast, showed very low guanine-nucleotide-binding activities. The number of bound nucleotide per Sar1p molecule was less than 0.1. Even with such low guanine nucleotide binding, E112K showed preferential binding of GDP to GTP$\gamma$S. Furthermore, the initial binding of GDP was rapid like that of the wild-type Sar1p. The GTP$\gamma$S binding at 30°C showed a slow and gradual increase with time. Because the E112K mutant is also temperature sensitive for growth (*24*), this could be due to nonspecific binding of GTP$\gamma$S to the denatured protein.

For a fixed period of time enough for saturation of the binding, varied amounts of guanine nucleotides were incubated with Sar1p and the bound fraction was quantitated by the same filter assay. From this experiment, the dissociation constant, $K_d$, and the number of nucleotide binding sites were determined by Scatchard plot. The results are shown in Table I. The kinetic parameters were not determined for the cases of GTP$\gamma$S binding with D32G and T54A, because they never reached saturation in the time scale we observed. Similarly, we were unable to obtain these values for E112K. Perhaps more elaborate methods such as equilibrium dialysis will be needed to assess these parameters.

The numbers of the nucleotide binding sites were all close to one per Sar1p molecule except for GTP$\gamma$S with D32G and T54A. The $K_d$ values of the wild-type Sar1p were determined to be 40 nM for GTP$\gamma$S and 60 nM for GDP at 20°C. These values are in a reasonable range for small GTPases. Both for the wild-type and mutant Sar1p, the $K_d$ values were higher at 30°C than at 20°C. The mutant proteins, especially H77L and C171S, showed significantly higher $K_d$ values than the wild-type. Considering the concentrations of guanine nucleotides in yeast cytosol, which are in the range of $10^{-4}$-$10^{-5}$ M (*12*), these lower affinities of the mutant proteins for guanine nucleotides will not affect the proportion of functional Sar1p. Indeed, as we reported previously (*23*), C171S behaves as a normal Sar1p *in vivo*. In the cases of D32G and T54A, the affinities for GTP$\gamma$S were too low to be determined by the filter assay. In other words, D32G and T54A can be regarded as mutants mostly fixed in the GDP-bound state.

*Intrinsic GTPase Activities and Stimulation by GAP*—The GTPase activities of the wild-type and mutant Sar1p were measured by incubating them with [$\alpha$-$^{32}$P]GTP and quantifying GTP and GDP after thin-layer chromatography. The results are shown in Table II. Among the wild type, H77L and C171S, there was no significant difference in the intrinsic GTPase activity. The turnover number of GTP hydrolysis was in the range of $2\text{-}4\times10^{-3}$ min$^{-1}$. This is in good agreement with the value previously reported for the wild-type Sar1p purified from yeast ($1.1\times10^{-3}$ min$^{-1}$) (*7*). D32G, T54A and E112K mutants, which do not bind GTP efficiently, also showed some ability to hydrolyze GTP. However, because we could not determine the numbers of the GTP (GTP$\gamma$S)-binding sites for these mutants, reliable estimation of the turnover number was impossible.

The effect of the addition of the Sec23p-Sec24p complex was also examined by this GTPase assay. As reported previously (*25*), Sec23p-Sec24p showed a GAP action on Sar1p and stimulated the GTPase activity of the wild-type Sar1p by 8.9-fold (Table II). Similar stimulation was also observed for C171S. In contrast, the GTPase activity of H77L was enhanced by only two-fold on the addition of Sec23p-Sec24p. This indicates that H77L is a mutant Sar1p that is much less sensitive to the action of GAP.

*Cell-Free Assays to Assess Sar1p Activity in ER-to-Golgi Vesicular Transport*—The purified wild-type and mutant Sar1p were further analyzed as to their abilities to promote ER-to-Golgi vesicular transport by cell-free assays. The system we utilized is based on the suppression of the temperature-sensitive transport of *sec12* semi-intact cells by the addition of functional Sar1p (*8, 22*). The *sec12* semi-intact cells were incubated at 27°C, the condition which is unable to promote vesicular transport to the Golgi in the absence of exogenously added Sar1p. When the wild-type Sar1p was supplied, the transport activity was restored and the marker protein, pro-$\alpha$-factor, acquired the *cis*-Golgi-specific $\alpha1\rightarrow6$ mannosyl modification on the *N*-linked oligosaccharides (Fig. 2). The amount of $\alpha1\rightarrow6$ mannosyl modification (1,700-2,000 cpm) upon the addition of 75 ng wild-type Sar1p was about 80-90% that for the same reaction at the permissive temperature (20°C). The C171S mutant also promoted transport to the Golgi. D32G appeared to have a weak activity to recover transport but the extent was much lower than the wild type. T54A and

TABLE I. **Guanine nucleotide binding of Sar1p mutants.**

| Sar1p | | $K_d$ (nM) | | Nucleotide binding site (pmol nucleotide/pmol Sar1p) | |
|---|---|---|---|---|---|
| | | GTP$\gamma$S | GDP | GTP$\gamma$S | GDP |
| WT | 30°C | 90±1 | 170±4 | 1.2±0.2 | 1.1±0.1 |
| | 20°C | 40±20 | 60±30 | 1.1±0.1 | 1.0 |
| D32G | 30°C | n.d. | 340±9 | n.d. | 1.2±0.1 |
| | 20°C | n.d. | 120±10 | n.d. | 1.1±0.1 |
| T54A | 30°C | n.d. | 160±1 | n.d. | 1.2±0.1 |
| | 20°C | n.d. | 80±20 | n.d. | 1.3±0.1 |
| H77L | 30°C | 650±160 | 640±90 | 0.85±0.26 | 0.9±0.1 |
| | 20°C | 200±80 | 190±10 | 0.85±0.19 | 0.83±0.02 |
| C171S | 30°C | 760±40 | 330±30 | 1.2±0.3 | 1.1 |
| | 20°C | 420±100 | 210±20 | 1.4±0.2 | 1.1 |

Purified Sar1p was assayed for [$^{35}$S]GTP$\gamma$S- and [$^{3}$H]GDP-binding as described in "MATERIALS AND METHODS." Values represent the means±SD for triplicate determinations. n.d., not determined.

TABLE II. **Intrinsic GTP hydrolysis activity of Sar1p and stimulation by Sec23p GAP.**

| Sar1p | GTP hydrolysis ($10^{-3}$ min$^{-1}$) | Stimulation by Sec23p (fold) |
|---|---|---|
| WT | 3.5±0.1 | 8.9 |
| H77L | 2.1±0.5 | 2.0 |
| C171S | 2.4±0.4 | 6.2 |

Purified Sar1p (0.1 $\mu$M GTP$\gamma$S-binding site) was incubated with 0.2 $\mu$M [$\alpha$-$^{32}$P]GTP (20,000 dpm/pmol) in the absence or presence of 0.1 $\mu$M Sec23p-Sec24p complex. GTP and GDP were separated on a polyethyleneimine thin-layer plate and analyzed by autoradiography. The values under GTP hydrolysis indicate the intrinsic GTPase activity in the absence of Sec23p-Sec24p. Mean values±SD for double or triplicate determinations are shown.

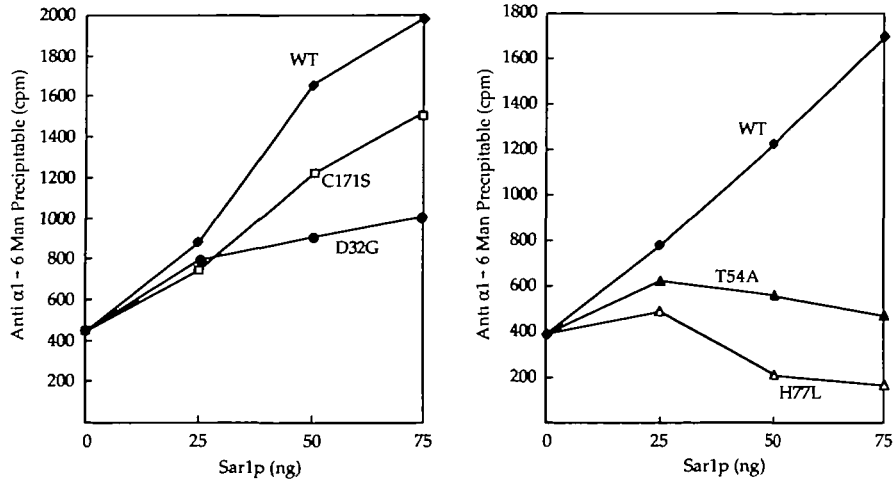


Fig. 2. **The activity of Sar1p to transport vesicles form the ER to the Golgi apparatus in a cell-free assay.** *sec12* semi-intact cells were incubated with the indicated amounts of purified Sar1p at 27°C for 60 min. The arrival of vesicles in the Golgi apparatus was assessed by the acquisition of $\alpha$1 →6 mannose linkage on pro-$\alpha$-factor as analyzed by immunoprecipitation (*22*).

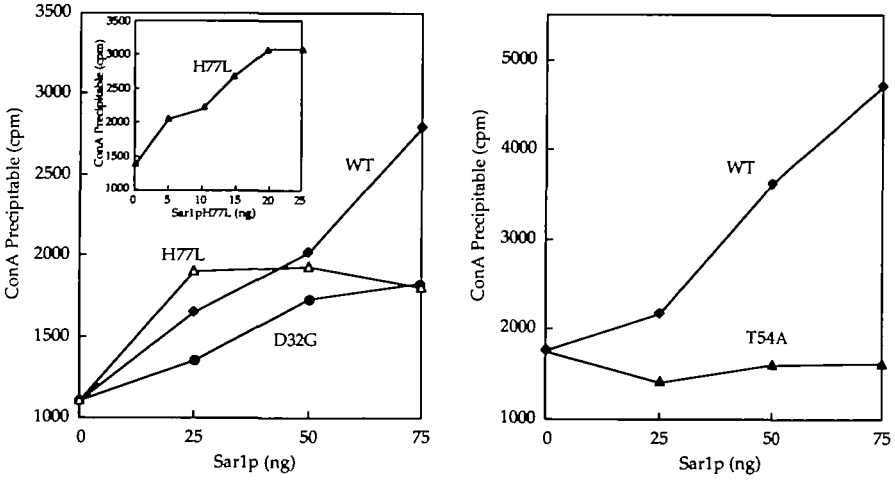


Fig. 3. **Vesicle formation activity of Sar1p from the ER membrane.** In a similar assay to that in Fig. 2, the ability of Sar1p to form vesicles was examined by collecting slowly-sedimenting membranes after the incubation of *sec12* semi-intact cells with Sar1p. The supernatant of the reaction mixture centrifuged at 20,000×*g* for 1 min was treated with trypsin and the pro-$\alpha$-factor in this vesicle fraction was quantified by precipitation with Con A-Sepharose (*8*).

H77L mutants showed virtually no transport activity.

In the sequential reactions of ER-to-Golgi transport, Sar1p is known to be required for the budding and formation of vesicles from the ER (*7, 8*). The vesicle formation can also be assayed using the semi-intact cell system by adding a medium-speed centrifugation step after the reaction to separate released vesicles (*8*). As shown in Fig. 3, the wild-type Sar1p promoted formation of vesicles as estimated by Con A-precipitable pro-$\alpha$-factor in the medium-speed supernatant. T54A, on the other hand, showed no activity of vesicle formation. D32G seemed to retain a little activity under these conditions. Interestingly, the H77L mutant showed a good vesicle formation activity at low concentrations. As shown in the inset of Fig. 3, Sar1pH77L caused a dose-dependent increase of vesicle formation up to 25 $\mu$g. At higher concentrations, however, H77L appeared to have a rather inhibitory effect.

To summarize, C171S showed a good activity in the overall transport from the ER to the Golgi. T54A could not support either vesicle budding or transport to the Golgi. D32G exhibited a very low activity to promote transport to the Golgi but the defect was already apparent at the vesicle formation step. The H77L mutant showed the ability to form vesicles at lower concentrations but no activity in the overall ER-to-Golgi transport reaction.

## DISCUSSION

As described in our previous paper (*23*), we introduced several point mutations into Sar1p, expecting to obtain mutant alleles that are somehow fixed at a particular stage of the GTPase cycle. In the case of Ras, detailed information is available as to which residue is important for this purpose. In fact, we designed the mutation points according to such information. Including the E112K mutation identified by a random screen for ts mutants (*24*), the mutations in Sar1p we now have are listed in Table III.

In this study, we purified five mutant proteins (D32G, T54A, H77L, E112K, and C171S) and examined their biochemical properties. The results are summarized in Table III. Interestingly, three of them, D32G, T54A, and E112K, showed preferential binding to GDP rather than to GTP. D32 in Sar1p corresponds to G12 in c-Ras-H, the position believed to be essential for the GTPase activity. In the case of Ras, mutations from G12 to any other residues cause the decrease of the GTPase activity and thus provide transforming activity (*26, 27*). Sar1p has D instead of G at this position to begin with, but the mutation from D to G apparently affects the affinity toward guanine nucleotides rather than GTP hydrolysis. In the case of yeast Arf1p, the

TABLE III. **Biochemical properties of mutant Sar1p.**

| Mutation in Sar1p | Corresponding mutation in c-Ras-H | Property of Sar1p mutant |
|---|---|---|
| GXXXXGK | | |
| D32G | G12 (This position is G in Ras) | Prefers GDP binding<br>Defective in vesicle budding |
| K36M | K16M | NT[a] |
| Effector domain | | |
| T54A | T35A | Prefers GDP binding<br>Defective in vesicle budding |
| DXXG(Q/H) | | |
| D73V | D57V | NT |
| H77L | Q61L | Normal GDP/GTP binding<br>Normal intrinsic GTPase<br>Insensitive to GAP<br>Can promote vesicle budding but not transport to the Golgi |
| NKXD | | |
| N132I | N116I | NT |
| Others | | |
| E112K | NA[a] | Low guanine nucleotide binding<br>Prefers GDP to GTP |
| C171S | NA | Almost normal |

[a]NT, not tested (mutant protein not purified); NA, not applicable (residue not conserved).

corresponding D26G mutant showed a defect in GTP$\gamma$S binding, too (*16*).

The property of T54A was also an unexpected one. This mutation was introduced into the PT motif, which is conserved in the effector domain of many small GTPases. Our results indicate that the T54A mutation fixes the Sar1p molecule in the GDP-bound state. This appears to be a quite unique example. It is known that the effector domain of c-Ras-H is important for the interaction with GAP (*28*). The T54A mutation in Rab3A has been shown to cause the decrease of its intrinsic GTPase activity and the inability to be activated by GAP and GEF (*29*). On the other hand, X-ray crystallographic data of c-Ras-H have shown that the corresponding T35 residue in Ras coordinates to $Mg^{2+}$ and the $\gamma$-phosphate of GTP (*30, 31*). The T54A mutation in Sar1p may have affected the interaction with not only GAP and GEF but also guanine nucleotides.

Sar1pE112K preferred GDP to GTP, but even for GDP, the binding activity was very low. In this case, we worried about the possibility that this mutant protein might have been partly inactivated during purification, because the *sar1*E112K mutant cells are also temperature sensitive for growth (*24*). We lowered the culture temperature of *E. coli* from 37 to 30°C but it did not improve the nucleotide-binding activities. We further tried to use a partially purified sample of this protein, which experienced a much shorter time of purification procedure, but we found similarly low activities of guanine nucleotide binding (unpublished results). One explanation for this may be that E112K has much lower affinity for GDP and GTP$\gamma$S than the wild-type, which resulted in the loss of the bound nucleotide during the wash of nitrocellulose filters. The observation that E112K can hydrolyse GTP would also argue against the possibility that the E112K protein was inactivated during purification.

H77L is also an intriguing mutant. The analogous Q61L mutation in Ras has been shown to exhibit low GTPase activity and again makes the protein transforming (*32*). In

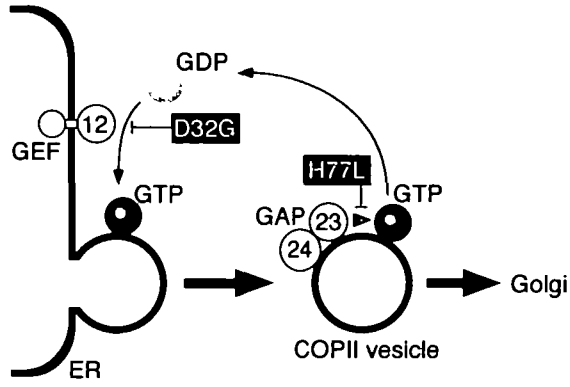


Fig. 4. **Model of the role of the GTPase cycle of Sar1p in vesicle formation.** Sar1p is depicted as shiny balls. The arrest points are shown for the cases of two representative mutants, D32G and H77L. 12, Sec12p; 23, Sec23p; 24, Sec24p.

the cases of Sec4p, Rab3A, and Rab5, similar Q-to-L mutations also caused the decrease of the intrinsic GTPase activity (*33–35*). On the other hand, the GTP hydrolysis by Sec4pQ79L and Rab3AQ81L was significantly stimulated by GAP. Our results for Sar1pH77L are quite different. The intrinsic GTPase activity of Sar1pH77L is comparable to that of the wild-type Sar1p and it is the sensitivity to GAP that is defective in this mutant. The Sec23p-Sec24p complex has the GAP activity towards the wild-type Sar1p and stimulates the low intrinsic activity by 8-10-fold. In contrast, the GTPase activity of H77L, which is slightly lower than that of the wild-type, is enhanced only by twofold by the addition of Sec23p-Sec24p. The stimulated GTPase activity is thus much lower than the case of the wild type. As a result, this mutation causes the stabilization of the GTP-bound state, which happened to be the same property as we expected from the knowledge on Ras proteins. A similar result, *i.e.* normal intrinsic GTPase activity and insensitivity to GAP, has also been reported in the case of yeast Ypt1pQ67L (*36*). It should be added that, even with such similar biochemical properties, Sar1pH77L and Ypt1pQ67L give totally different phenotypes in transport. Sar1pH77L is very toxic and stops ER-to-Golgi transport of wild-type cells in a dominant fashion (*23*). In contrast, the hydolysis of GTP by Ypt1p does not seem to be required to drive the same transport step *in vivo* or *in vitro*, and in fact Ypt1pQ67L can replace the wild-type Ypt1p function (*36*).

The C171S mutant showed almost normal properties as compared to the wild type. It has somehow lower affinities to guanine nucleotides, but apparently this does not affect its *in vivo* phenotype. The yeast cells that depend on *SAR1*C171S grow normally (*23*). The purpose of this mutation was to examine the possibility of modification on the single cysteine residue of Sar1p, which might be important for the function. As mentioned in the introduction, we have already concluded that the answer is no.

Cell-free transport assays were performed to examine the abilities of these mutant proteins to drive ER-to-Golgi transport. Using a non-hydrolyzable analogue of GTP, GTP$\gamma$S, we previously demonstrated that the hydrolysis of GTP is not required for the budding and formation of vesicles from the ER but is essential for progress to the step of targeting to the Golgi apparatus (*8*). This can now be

interpreted as that the conversion of the active GTP-bound Sar1p to the inactive GDP-bound Sar1p leads to the disassembly of the coat protein complex (COPII) (*18*) from the vesicles, and thus exposes the machinery required for vesicle targeting and fusion. Our results for the H77L mutant support this model. The H77L protein can drive vesicle formation in our cell-free assay. However, it showed no detectable ability to transport vesicles to the Golgi apparatus. It should also be noted that high concentrations of H77L cause the inhibition of vesicle formation. This result is similar to that we obtained previously with the wild-type Sar1p complexed with GTP$\gamma$S (*8*). Presumably, the consumption of GTP is needed for the multiple rounds of vesicle formation and thus its inhibition eventually causes the budding reaction to plateau.

Our model for the role of the GTPase cycle of Sar1p in vesicle formation is shown in Fig. 4 together with the effects of two representative mutations, D32G and H77L. This model is also consistent with our *in vivo* observations. The mutant cells with *sar1*D32G are temperature sensitive for growth. Upon a shift up to the restrictive temperature, the cells accumulate a massive amount of ER membrane but no appreciable vesicles (*24*). In contrast, when the secretion of yeast cells is arrested by the overexpression of *SAR1*H77L, some vesicular or tubular structures are observed in addition to the exaggerated ER membrane (A. Hirata, T. Oka, and A. Nakano, unpublished).

Knowing their biochemical properties, these mutants will be extremely useful for identifying the regulatory components of the Sar1p GTPase cycle. As described by Nakano *et al.* (*23*), most of the mutations show a dominant negative effect toward the wild type when they are overexpressed. D32G and E112K are also very useful because they are recessive and temperature sensitive when they are expressed from a single-copy plasmid. Suppressor screens have been performed in a variety of ways and some results will be reported elsewhere.

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