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Characterization of Yeast *sar1* Temperature-Sensitive Mutants, Which Are Defective in Protein Transport from the Endoplasmic Reticulum[1]

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***SAR1* encodes a low molecular weight GTPase that is essential in the early process of vesicular transport in the secretory pathway. By random and site-directed mutagenesis of the *SAR1* gene, we have obtained three temperature-sensitive mutants, N132I, E112K, and D32G. They all show a defect in transport from the endoplasmic reticulum to the Golgi apparatus, and accumulate endoplasmic reticulum membranes at the restrictive temperature. This is consistent with our previous observations *in vivo* on a galactose-shutoff mutant as well as the *in vitro* results, and provides powerful tools for further genetic analyses.**

Key words: Golgi apparatus, protein secretion, small GTPase, *Saccharomyces cerevisiae*, vesicular transport.

Studies in the last decade on intracellular transport of proteins have revealed that a large number of low molecular weight GTPases are involved in a variety of steps of vesicular traffic in eukaryotic cells (for a review, see Ref. *1*). The product of the *SAR1* gene, Sar1p (*2*), plays a pivotal role in the formation of transport vesicles from the endoplasmic reticulum in yeast (*3, 4*) and mammalian cells (*5*), and probably in plant cells too (*6*). Another subfamily of small GTPases, Arf, is involved in vesicle formation from the Golgi membrane (see Ref. *7*). The Sar and Arf families are structurally related to each other but distant from the Ypt/Rab family, which is believed to function in the targeting and/or fusion of vesicles with acceptor membranes. To understand the molecular basis of Sar1p functions in detail, we performed site-directed mutational analysis of the yeast *SAR1* gene, and identified several dominant lethal alleles and two conditional temperature-sensitive (ts) alleles (N132I and D32G) (*8*). Among them, the ts alleles are extremely useful for analysis of the role of the Sar1p GTPase cycle *in vivo*. In this paper, we report the isolation of a new ts allele (E112K) by random mutagenesis and characterization of the three ts alleles with respect to the role of Sar1p in ER-to-Golgi protein traffic.

MATERIALS AND METHODS

Strains and Culture Conditions—The *Escherichia coli* strains used for recombinant DNA work in this study were DH5α (*supE44 ΔlacU169* [ϕ80 *lacZΔ*M15] *hsdR17 recA endA1 gyrA96 thi-1 relA1*), SCS1 (F$^-$ *endA1 hsdR17* [γ_κ^-, m_κ^+] *supE44 thi-1 recA1 gyrA96 relA1*), and XL1-Blue (*supE44 hsdR17 recA endA1 gyrA46 thi-1 relA1 lac*$^-$ F′ [*proAB*$^+$ *lacI*^{q}*lacZΔ*M15 Tn*10*(*tet*^{r})]). The *Saccharomyces cerevisiae* strains used in this study are listed in Table I. Yeast cells were grown in YPD [1% (w/v) Bacto yeast extract (Difco, Detroit, MI, USA), 2% (w/v) polypeptone (Nihon Seiyaku, Tokyo), and 2% (w/v) glucose] or MVD [0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose] supplemented appropriately. For derepression of the *GAL1* promoter, YP or MV medium was supplemented with 5% (w/v) galactose and 0.2% (w/v) sucrose (YPGS or MVGS). The growth of cells was monitored by measuring A_{650} with a Coleman spectrophotometer (Coleman, Heywood, IL, USA). One A_{650} unit corresponded to 5×10^7 cells/ml approximately.

Plasmids and DNA Manipulations—Yeast plasmids, pANY2-7, pANY2-18 (*2*), and the pMYY3 series (*8*), have been described previously. pMYY3-1, pMYY3-5, pMYY3-7, and pMYY3-9 are low copy number plasmids containing the wild type, N132I, D32G, and E112K versions of *SAR1* cDNA, respectively. pTY3 is a low copy number plasmid containing the genomic version of the wild type *SAR1* gene, and was constructed by inserting the 1.4 kb *Hin*dIII/*Sal*I fragment from pANY2-7 into the multicloning sites of pRS315 (*9*) and creating an *Nco*I site at the initiator ATG codon of *SAR1* by site-directed mutagenesis (see Ref. *3*). pMYT3-7 contains the D32G version of *SAR1* cDNA in pTY3. pTY5 was constructed by insertion of the *GAL1* promoter into the *Bam*HI site in front of the wild-type *SAR1* gene in pTY3 (see Ref. *2*).

DNA manipulations including restriction enzyme digestions, ligation, plasmids isolation, and *E. coli* transformation were carried out by standard methods. Yeast transformation was performed by the lithium thiocyanate method

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Abbreviations: ER, endoplasmic reticulum; CPY, carboxypeptidase Y.

TABLE I. **Yeast strains used in this study.**

Strains	Genotypes
ANY25[a]	*MATa Δsar1::URA3 ura3 leu2 trp1 his* [pANY2-18 (YCp*GAL1-SAR1 TRP1*)]
ANY26[a]	*MATα Δsar1::URA3 ura3 leu2 trp1 his* [pANY2-18 (YCp*GAL1-SAR1 TRP1*)]
ANY27[b]	*MATa Δsar1::URA3 ura3 leu2 trp1 his* [pTY5 (YCp*GAL1-SAR1 LEU2*)]
ANY29[c]	*MATa Δsar1::URA3 ura3 leu2 trp1 his* [pTY3 (YCp*SAR1 LEU2*)]
TOY224[c]	*MATα ura3 leu2 trp1 his3 ade2 lys2 Δsar1::HIS3 pep4::ADE2* [pMYY3-9 (YCp*sar1*E112K *TRP1*)]
TOY410[c]	*MATa ura3 leu2 trp1 his3 ade2 lys2 sar1-3::TRP1*

References: [a](*2*); [b](*8*); [c]this study.

(*10*). DNA fragments were purified from agarose gel pieces using the DNA PREP kit (Asahi Glass, Tokyo). DNA nucleotide sequences were determined by the dideoxy method using the Sequenase kit (United States Biochemical, Cleveland, OH, USA). Site-directed mutagenesis was carried out using the Oligonucleotide-directed *in vitro* mutagenesis system (Amersham, Arlington Heights, IL, USA).

Random Mutagenesis of Plasmid and Mutant Screening—Plasmid pTY3 was treated with hydroxylamine according to the method of Schauer *et al.* (*11*). Briefly, 125 μg/ml plasmid DNA was incubated in 0.4 M hydroxylamine-HCl and 0.05 M potassium phosphate (pH 6.0) at 75°C for 1 h, and then cooled on ice. After the addition of 1/20 volume of 3 M sodium acetate (pH 4.8), DNA was precipitated three times with ethanol, dried well, and dissolved in 10 mM Tris-HCl and 1 mM EDTA (pH 8.0). ANY25 yeast cells were transformed with this mutagenized DNA and spread on MVGS (-leu) plates to yield 100–200 colonies per plate. After several days at 30°C, the colonies were replica-plated onto two sets of YPD plates, which were then incubated for an additional several days at 23 and 37°C (Fig. 1). The colonies that grew at 23°C but not at 37°C were picked up, restreaked on YPD to confirm the ts growth, and then subjected to further analysis. Plasmid DNA was recovered from the candidate yeast cells, amplified in *E. coli* and examined for the restriction patterns. The plasmids derived from pTY3, which were originally mutagenized and should therefore carry mutations, were reintroduced into ANY25 and examined as to whether they reproduce the ts growth phenotype on glucose. One of the recovered plasmids, pRME12, showed a ts nature in the retransformation. The *Nco*I/*Eco*RI fragment containing the whole ORF of the *SAR1* gene (harboring the "E112K mutation"; see "RESULTS") was excised from pRME12 and inserted into the original pTY3 and pMYY3-1. These exchanged plasmids (pRME12-0 and pRME12-1, respectively) conferred ts growth again. To make the cDNA version of this mutant gene, the 0.5 kb *Nde*I/*Eco*RI fragment of pMYY3-1 was replaced by the corresponding fragment from pRME12, that contained the mutation (pMYY3-9). DNA sequences were confirmed for the presence of the mutation in all of the plasmids used in this study.

Immunological Methods—Immunoblotting and immunoprecipitation were performed as previously described (*2, 12*). The rabbit polyclonal antibody against carboxypeptidase Y was prepared and used as described before (*2*).

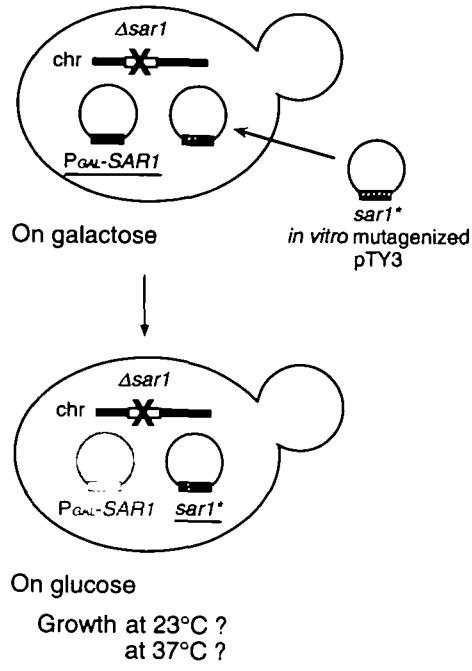


Fig. 1. **Strategy for the isolation of temperature-sensitive *sar1* mutants.** Yeast cells whose growth depends on the *GAL1*-promoter-driven expression of *SAR1* on a plasmid (P_{GAL}-*SAR1*) were transformed with a library of mutagenized *SAR1* plasmid (*sar1**). Cells that showed temperature-sensitive growth upon shift to glucose medium were selected. For details, see under "MATERIALS AND METHODS."

Electron Microscopy—The preparation of thin sections of yeast cells by the freeze-substituted fixation method was carried out as described (*13*). Observations were made with a JEOL 200CX electron microscope (JEOL, Tokyo) at 100 kV.

RESULTS

Isolation of a New ts Allele of SAR1 by Random Mutagenesis—As we described in our previous paper (*8*), site-directed mutagenesis of the *SAR1* gene has unveiled two mutations, Asn to Ile at position 132 (N132I), and Asp to Gly at position 32 (D32G), that confer ts growth on the cells. We wished to obtain more conditional alleles of *SAR1* that give a similar ts phenotype and attempted random mutagenesis *in vitro*. A low copy number plasmid (pTY3) containing the genomic wild-type *SAR1* gene was treated with hydroxylamine to induce C-to-T mutations in a random fashion. The plasmid DNA containing a library of mutated genes was introduced into ANY25 cells, which have the chromosomal *SAR1* gene deleted but harbor the wild-type *SAR1* gene under control of the *GAL1* promoter (see Fig. 1). The transformants obtained on minimal galactose plates were replica-plated onto two sets of glucose plates, which were then incubated at 23 and 37°C. From the colonies that appeared to grow at 23°C but not at 37°C, the mutated pTY3 plasmid was recovered and reintroduced into ANY25 cells to examine whether this plasmid confers the ts nature again. Among the 50,000 colonies we screened, one plasmid gave the ts phenotype reproducibly and was characterized further.

The New ts Allele has the E112K Mutation—The recovered plasmid was purified and the sequence of the whole region of the *SAR1* gene was determined. There was only one mutation, G-to-A, at nucleotide position 334 of the *SAR1* ORF, which resulted in the Glu-to-Lys mutation at amino acid position 112 (E112K) (see Fig. 2). Confirmation

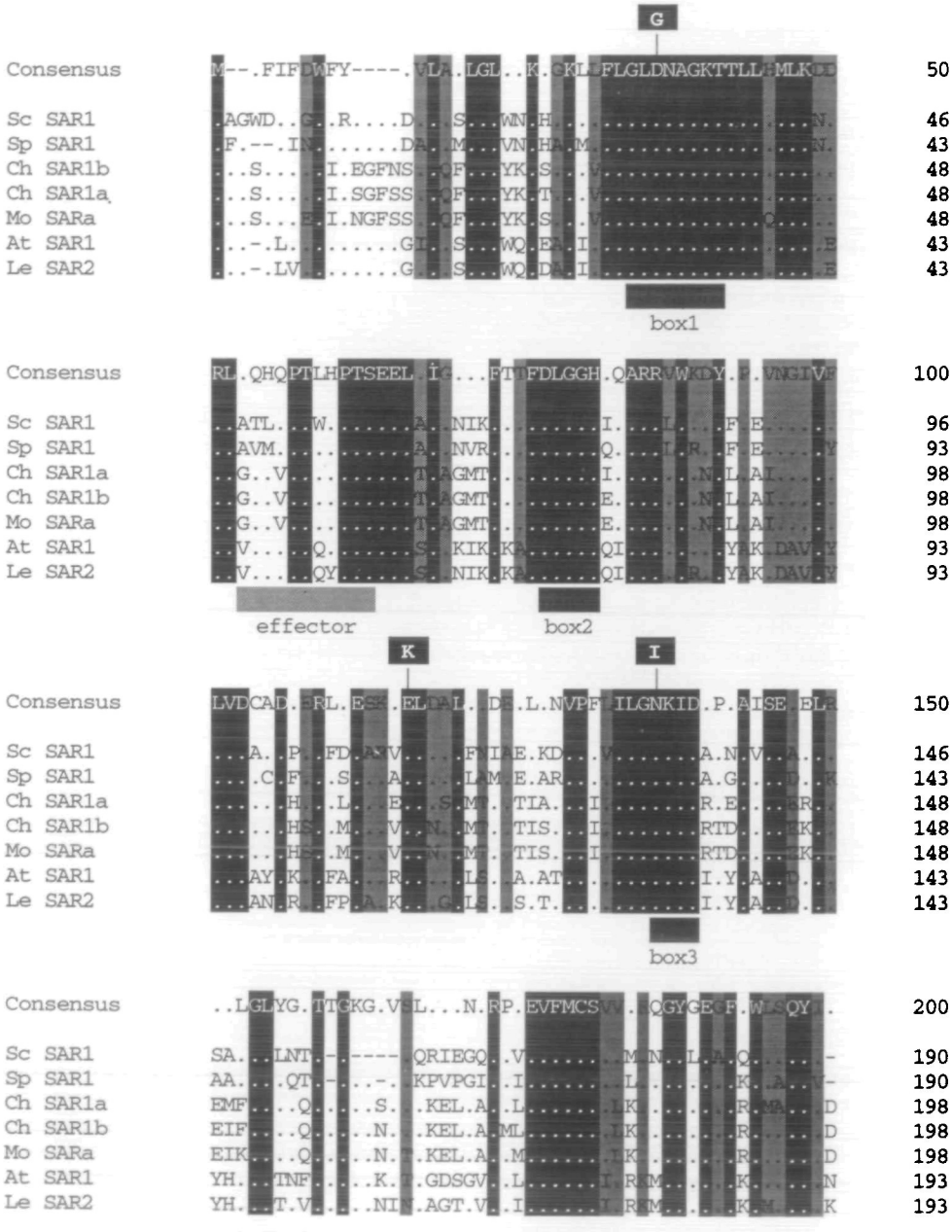


Fig. 2. **The mutation points of the three ts alleles in the amino acid sequence of Sar1p.** A comparison of Sar1p sequences from different organisms is also shown. The completely and highly conserved residues are shadowed in black and gray, respectively. Sc, *Saccharomyces cerevisiae* (X51667); Sp, *Schizosaccharomyces pombe* (M95797); Ch, Chinese hamster ovary cells (559644, 559645); Mo, mouse AtT-20 cells (L20294); At, *Arabidopsis thaliana* (M95795); Le, *Lycopersicon esculentum* (tomato) (L12051). The numbers in parentheses are the database accession IDs of GenBank.

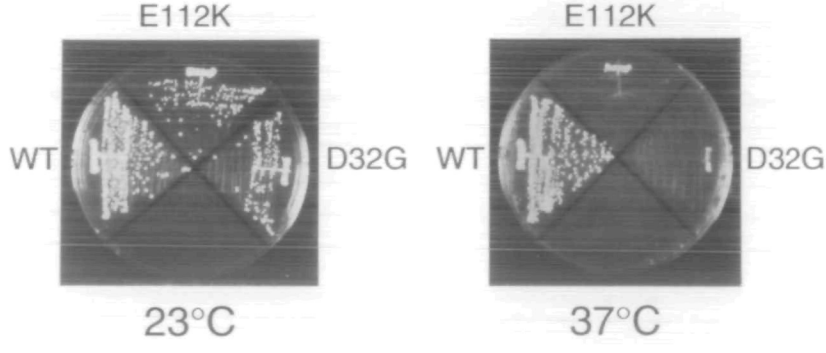


Fig. 3. **Growth of the newly obtained *sar1* ts mutant on plates.** ANY27/pRME12-1 (E112K) cells were streaked on YPD plates and incubated at 23 and 37 C for 4 days. The growth of ANY27/pMYY3-1 (wild type) and ANY27/pMYY3-7 (D32G) cells is also shown.

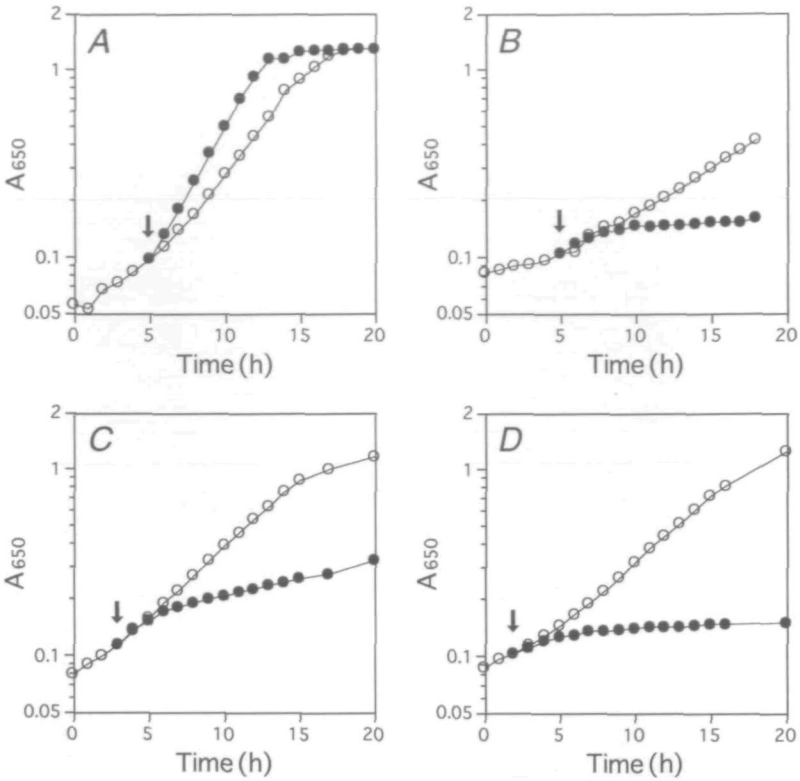


Fig. 4. **Growth curves of the three *sar1* ts mutants in liquid culture.** Wild-type (*A*, ANY27/pMYY3-1), N132I (*B*, ANY27/pMYY3-5), E112K (*C*, ANY26/pRME12-0), and D32G (*D*, ANY26/pMYT3-7) cells were inoculated into YPD medium, cultured at 23°C until A_{650} reached about 0.1, divided into two, and further incubated at 23 (○) and 37°C (●). The arrows indicate the point of temperature shift.

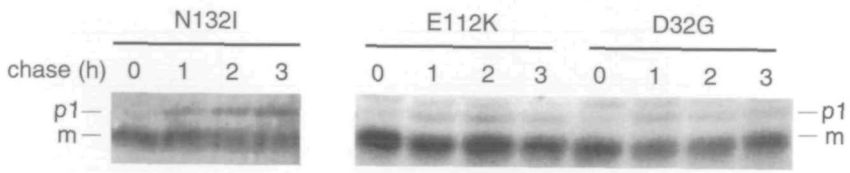


Fig. 5. **Immunoblotting analysis of CPY in *sar1* ts mutants.** N132I (ANY27/pMYY3-5), E112K (ANY26/pRME12-0), and D32G (ANY26/pMYT3-7) cells were cultured in YPD at 23°C to the early log phase and transferred to 37°C. At 0, 1, 2, and 3 h after the temperature shift, aliquots were withdrawn, and subjected to SDS-PAGE and immunoblotting with the anti-CPY antibody. p1, ER-precursor form; m, mature form.

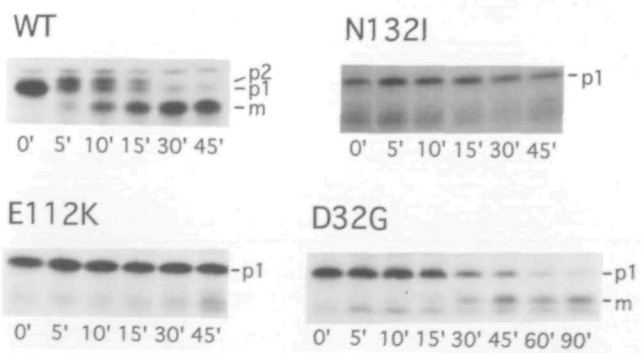
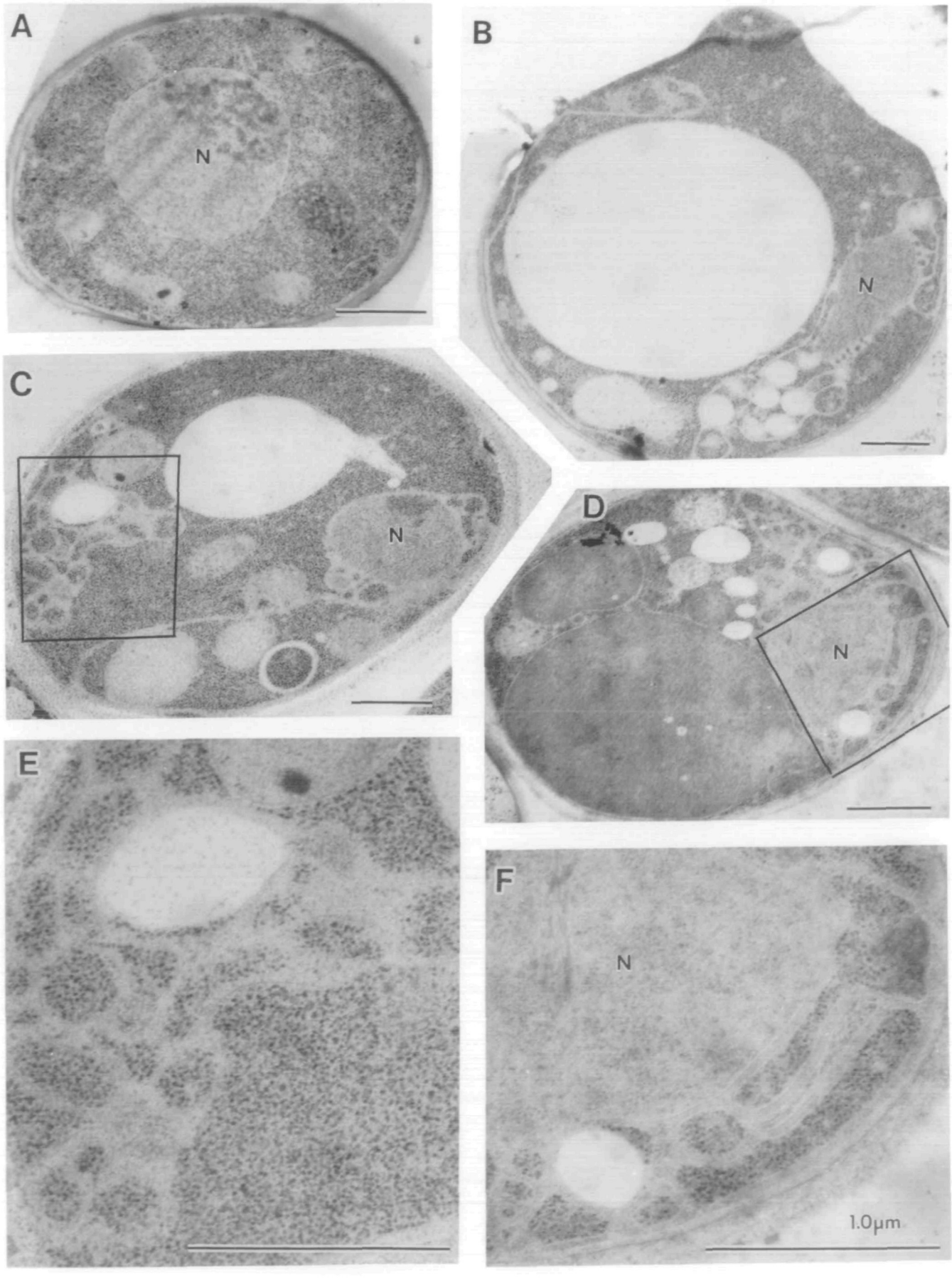


Fig. 6. **Pulse-chase experiments to follow the intracellular transport of CPY.** Wild-type (ANY29), N132I (ANY27/pMYY3-5), E112K (TOY224), and D32G (TOY410) cells were grown to the early log phase at 23°C, incubated at 37°C for 15 min, labeled with Tran^{35}S-label for 5 min, and chased at 37°C. Aliquots were withdrawn at the indicated times and subjected to sampling for immunoprecipitation with the anti-CPY antibody. p1, ER-precursor form; p2, Golgi-precursor form; m, mature form.

that this is the mutation that causes the ts phenotype was performed by replacement of the whole ORF of the wild-type gene by that of the mutant. The temperature-sensitive growth of this newly obtained E112K mutant on YPD plates is shown in Fig. 3. Together with those obtained by site-directed mutagenesis, we now have three ts alleles of *SAR1*. According to the order of isolation, the N132I, E112K, and D32G alleles were named *sar1-1*, *sar1-2*, and *sar1-3*, respectively. To avoid confusion, however, we will always indicate the mutations as E112K *etc.* in this paper.

Growth Phenotypes of the sar1 ts Mutants—The growth curves of the three ts mutants in liquid YPD medium are shown in Fig. 4. They all showed a rapid ts block in growth. The N132I mutant cells appear to express a defect even at low temperatures; their growth at 23°C was much slower than that of the wild-type cells (Fig. 4B). Like other *sec* mutants, the viability of the cells decreases quickly at the restrictive temperature. In the case of E112K, for example, the proportion of viable cells decreased to about 50% when the culture was incubated at 37°C for 2 h.

The E112K ts mutation is recessive to the wild type, like the other two mutations. When two low-copy-number plasmids of the same type containing the wild-type and mutant genes are present, the cells grow at 37°C (data not shown). On the contrary, E112K shows a dominant lethal effect when the mutant gene is overexpressed by the *GAL1* promoter in the wild-type cells (data not shown). This is also a common property of the three ts mutations (see Ref.



8). These two distinct phenotypes will be useful for further genetic analysis.

The sar1 ts Mutants Are Blocked in ER-to-Golgi Protein Transport—Previously, we showed that the *SAR1* gene is essential for growth, and that the shutoff of its expression causes a defect in the ER-to-Golgi transport of secretory and vacuolar proteins (*2*). This study was performed with a galactose-dependent conditional-lethal mutant of *SAR1*, the same strain we used for the mutant screening in this study. The disadvantage of *GAL1* shutoff experiments is that a long time is required for expression of the mutant phenotypes because the wild-type gene product that is overproduced on galactose has to be turned over before the cell shows the deficiency. In the case of the *GAL1-SAR1* gene construct, 12–15 h were needed to see the phenotype after the cells had been shifted to the glucose medium.

A great merit of ts mutants is that the block is usually imposed immediately after the shift to the restrictive temperature. We tested the role of Sar1p in ER-to-Golgi traffic with these ts mutants, using a vacuolar protein carboxypeptidase Y (CPY) as a marker of transport. When the mutant cells were shifted to 37°C, accumulation of the ER-precursor form (p1) of CPY was detected by immunoblotting within 1 h (Fig. 5). This indicates that the normal function of Sar1p is required for ER-to-Golgi transport, as shown by our previous work on the galactose-dependent mutant.

The transport defects of these mutant cells were further examined by pulse-chase experiments (Fig. 6). In the wild type cells (WT), the conversion from the ER-precursor (p1) through the Golgi-form (p2) to the mature CPY takes place almost completely within 30 min. In contrast, N132I and E112K ts mutants showed a clear block in p1 to p2 conversion of the CPY precursors, again indicating the essential role of Sar1p in protein transport from the ER. In the case of D32G, the block was not complete. However, the p1 form persisted much longer than the wild type. Even after a 45 min chase, nearly half of CPY still existed as the p1 form. The p2 form was hardly detected. These findings indicate that the ER-to-Golgi transport is rate limiting in this mutant.

The sar1 ts Mutants Accumulate ER Membranes—The morphological alterations of the mutant cells were examined by electron microscopy. The wild-type and mutant cells were incubated at 37°C for 2 h and then processed for freeze substitution electron microscopy. As shown in Fig. 7, it is eminent that all of the ts mutants exaggerate ER membranes, consistent with the biochemical results as described above. In most pictures we examined, the mutant cells did not show remarkable accumulation of vesicles.

DISCUSSION

The three ts mutations obtained are mapped distantly in the amino acid sequence of Sar1p. As shown in Fig. 2, D32 is in the first consensus box of the GTP-binding site and N132 is in the third box. The specific role of E112 is not known, but this residue is completely conserved in the *SAR1* gene family and is present in most Arfs, but not in others. Three-dimensional modeling of Sar1p by means of Swiss-Model (available through the World Wide Web at the internet address, "http://expasy.hcuge.ch/") based on the X-ray structure of human Arf1 (*14*) predicts that D32 and E112 are actually located close in space. In fact, the recombinant D32G and E112K proteins purified from *E. coli* showed defective binding of GTP (Kimura, Saito, Oka, and Nakano, manuscript in preparation), suggesting that their phenotypes are due to a similar defect, namely, arrest of the GTPase cycle in the GDP-bound state (see below). By analogy to many other members of the Ras superfamily, N132 must be important for guanine ring recognition (*15*), and the N132I mutation probably has lower affinities to GDP and GTP. Unfortunately, the N132I protein was not stably expressed in *E. coli* cells. Its biochemical properties remain to be determined.

It should be noted that these ts mutations have been obtained by procedures based on plasmids. We tried to integrate the individual mutations into the yeast chromosome but were successful only in the case of the D32G allele. If E112K is on the low copy number plasmid, it can sustain cell growth at 23°C without the wild type copy of the *SAR1* gene. Perhaps a little elevated level of the gene dosage (the low copy number plasmids of yeast can exist in 2–3 copies) is necessary for the E112K mutant to survive. On the other hand, N132I was never able to support cell growth solely by itself. To see the ts phenotype of N132I, another plasmid containing the wild type *SAR1* gene under control of the *GAL1* promoter had to coexist (see Ref. *8*). Even on glucose, a tiny expression of the wild-type *SAR1* gene may be leaking from the *GAL1* promoter regulation, which probably facilitates the N132I-dependent cell growth at the low temperature. In accord with these observations, we were not able to obtain any ts alleles of the *SAR1* gene by *in vivo* mutagenesis. We also admit that our attempts to isolate ts alleles are not saturating, because neither D32G nor N132I was fished out in our screening that was based on plasmid procedures. Further extensive screening might reveal other conditional mutations of the *SAR1* gene.

By establishing cell-free transport assays, we have shown that the GTPase cycle of Sar1p is essential for multiple rounds of vesicle formation and targeting in ER-to-Golgi traffic (*3, 4*). If Sar1p is locked in the GDP-bound state by the ts defect of Sec12p (guanine nucleotide exchange factor for Sar1p), vesicle formation does not occur. If Sar1p is fixed in the GTP-bound state by the use of GTPγS, vesicles are formed from the ER but not targeted to the Golgi. From these and other observations, we have proposed the following model. Sec12p catalyzes GDP-to-GTP exchange on Sar1p, which then triggers the assembly of coat proteins (COPII; *16*) onto the ER membrane and budding of vesicles. When the vesicle formation is completed, Sec23p, a GTPase-activating protein for Sar1p, stimulates the GTP hydrolysis by Sar1p. Sar1p-GDP is dissociated from the vesicles together with other coat proteins, and the uncoated vesicles now become competent for targeting to the Golgi. In these processes, at which stage of transport is each of the *sar1* ts mutants blocked?

Fig. 7. **Electron microscopic observation of the *sar1* ts mutants.** Wild-type (ANY29), N132I (ANY27/pMYY3-5), E112K (TOY224), and D32G (TOY410) cells were grown to the early log phase at 23°C, incubated at 37°C for 2 h, and then subjected to rapid freeze substitution. The fixed cells were embedded in Spurr's resin, thin-sectioned, and observed under an electron microscope. A, wild type; B, N132I; C, E112K; D, D32G; E, enlargement of C; F, enlargement of D. Bar, 1.0 μm.

From electron microscopic observations, it is obvious that all the three ts mutants pile up ER membranes. The important point is whether these mutants accumulate transport vesicles as well. In most sections we examined, the presence of vesicles was not very much exaggerated, at least in the E112K and D32G mutants. This may be reasonable if they are in fact locked in the GDP-bound state at the restrictive temperature. The *sec12* ts mutant, which is defective in GDP-to-GTP exchange, accumulates massive amounts of ER membranes but not vesicles. On the other hand, N132I might show a different phenotype. If its affinities to GDP and GTP are similarly reduced as is the case of Ras and other GTPases, the mutant protein may exist mostly complexed with GTP since the cytosolic pool of GTP is much larger than that of GDP. It is not evident whether such a property could lead to the accumulation of vesicles as was seen in the cell-free system when Sar1p was complexed with GTPγS. Unfortunately, the N132I mutant cells are very sick at the restrictive temperature and we could not draw a convincing conclusion. More elaborate examination will be necessary to address this problem. Cell-free assays are also very important for analysing vesicle formation and targeting in these mutants, and will be published elsewhere.

It may also be worth mentioning here that the ER membranes that accumulate in the E112K and D32G mutants are not in direct contact with ribosomes (see Fig. 7, E and F). It looks as though the membranes are coated to prevent ribosome attachment. This smooth ER-like structure is also observed when other ER-to-Golgi *sec* mutants are incubated at the restrictive temperature (Hirata and Nakano, unpublished results).

These *sar1* ts mutations are not only helpful for the biochemical analysis of protein traffic, but also provide alluring possibilities for yeast genetics. A variety of genetic means can be applied to find novel components that regulate the Sar1p function. Multicopy suppressors, extragenic suppressor mutations, and mutations that are lethal when combined with *sar1* ts may all lead to the identification of missing factors that play roles in the Sar1p GTPase cycle. Great efforts are now being made along these lines in our laboratory. We have recently found a novel gene that can suppress the ts defects of E112K and D32G on a multicopy plasmid (Saito, Yamanushi, Oka, and Nakano, unpublished). Detailed analysis of this and other suppressor genes will facilitate understanding of the essential function of Sar1p, and will be reported elsewhere.

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