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Identification and Characterization of Extragenic Suppressors of the Yeast *sec12* ts Mutation[1]

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The product of the yeast *SEC12* gene is the most upstream player as far as we know in the events of vesicle budding from the endoplasmic reticulum membrane. To understand how the Sec12p function could be regulated, I identified three extragenic suppressor mutations of the *sec12* ts mutation. *RST1*^{DS} is a dominant mutation and causes elevated expression of Sec12p. *rst2* and *rst3* are recessive and give pleiotropic phenotypes including slow growth at low temperatures and heterogeneous modification of Sec12p. Their possible roles are discussed.

Key words: endoplasmic reticulum, *RST*, *Saccharomyces cerevisiae*, *SEC12*, vesicular transport.

The yeast *SEC12* gene encodes an integral membrane protein which resides in the endoplasmic reticulum (ER) membrane (*1*). Its role was originally identified by a temperature-sensitive (ts) mutation that blocks transport of secretory, vacuolar, and plasma membrane proteins from the ER to the Golgi apparatus at the restrictive temperature (*2-4*). I isolated *SAR1*, a gene encoding a low molecular weight GTPase, as a multicopy suppressor of the *sec12* ts mutant (*5*). Our subsequent analyses mainly by cell-free reconstitution experiments and also studies by Schekman's group have demonstrated that the gene products of *SEC12* and *SAR1* play essential roles in the initial events of vesicle formation from the ER membrane (*6-12*). Sec12p is now known to possess a guanine nucleotide exchange activity towards Sar1p (*13*) and is thus regarded as the most upstream player in the budding reaction. Once Sec12p triggers the activation of Sar1p, peripheral membrane proteins collectively called COP II (*12*) assemble on the ER membrane and formation of a vesicle is promoted.

Sec12p has also been an object of studies on localization mechanisms of ER membrane proteins. It is a type II integral membrane protein with a single membrane span, the N-terminal domain facing the cytoplasm. We have shown that it is localized to the ER by two mechanisms: static retention in the ER and dynamic retrieval from the Golgi apparatus. We have also identified the *RER* genes that are involved in these mechanisms (*14, 15*). Our most recent study has elucidated the structural requirements of Sec12p for ER localization (*16*). The transmembrane domain of Sec12p acts as a retrieval signal, whereas the cytoplasmic domain has a signal for static retention.

Although such a large body of information is accumulating on Sec12p, the regulation of its function is still unknown. In the budding events from the ER, there must proceed sorting that discriminates proteins to be packaged into the vesicles from those to be left behind. It is quite conceivable that such sorting information or a signal would be transduced from the lumenal side of the ER to the budding machinery. The Sec12p molecule would be a good candidate as a transceiver of such a signal.

In the hope of identifying cellular components that regulate the Sec12p function, I conducted a genetic approach. I found three mutations that suppress the temperature sensitivity of the *sec12-4* mutation. Their characterization will be described in this paper.

MATERIALS AND METHODS

Strains—Yeast strains used in this study are shown in Table I. The cells were grown in the YPD medium (1% yeast extract, 2% polypeptone, and 2% glucose).

Immunoblotting Analysis—Preparation of cell lysates, SDS-PAGE, and immunoblotting were performed as described previously (*5*). The antibodies against Sec12p (*1*), Sar1p (*5*), and carboxypeptidase Y (*5*) were used.

RESULTS

Isolation of Revertants of sec12—The *sec12-4* mutant cells show tight temperature sensitivity in growth. They grow very slowly at 30°C and completely stop growth at 33°C. I tried to collect spontaneous revertants of this mutant that grow at 37°C. I streaked approximately 1×10^8 *sec12-4* cells (MBY10-7A) on a YPD plate and incubated it at 37°C. This gave rise to 46 colonies, which I patched on a new YPD plate, kept at 37°C overnight and replicated to three plates, which were further incubated at 37, 24, and 15°C. Three clones showed growth at 37°C (Ts^+) but no or very slow growth at 15°C (Cs^-) (see Fig. 1). I picked up these Cs^- clones because this phenotype would be a useful marker for further analysis, and named them STR1, STR2, and STR3. The Cs^- nature of STR2 and STR3 was quite

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

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

Strains	Genotypes
MBY10-7A[a]	*MATa sec12-4 ura3 leu2 trp1 his3 his4*
MBY10-7C[a]	*MATα sec12-4 ura3 leu2 trp1 his4*
ANY21[b]	*MATa ura3 leu2 trp1 his3 his4*
JRY9[b]	*MATα ura3 leu2 trp1 his3 his4*
STR1[c]	*MATa RST1[DS]-1 sec12-4 ura3 leu2 trp1 his3 his4*
STR2[c]	*MATa rst2-1 sec12-4 ura3 leu2 trp1 his3 his4*
STR3[c]	*MATa rst3-1 sec12-4 ura3 leu2 trp1 his3 his4*

References: [a](*1*); [b](*5*); [c]this study.

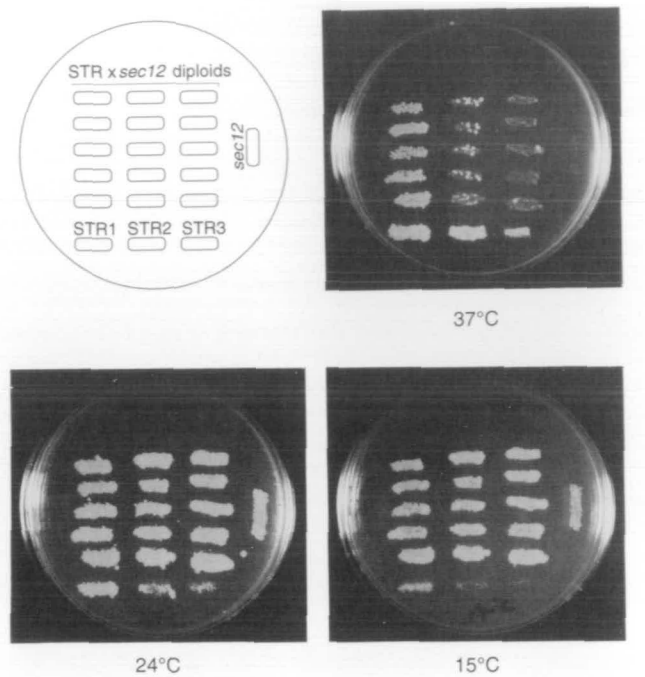

Fig. 1. **Growth of spontaneous revertants of the *sec12* ts mutant.** Three clones (STR1, STR2, and STR3) derived from MBY10-7A (*sec12*) that show Ts$^+$ and Cs$^-$ phenotypes were selected. The diploid cells (five isolates each) made by mating STR1, STR2, and STR3 with MBY10-7C (*sec12*) are also shown.

severe. Even at 24°C, their growth was much slower than the parent *sec12* or the wild-type cells.

Genetic Analysis—First, these three revertants were mated with the *sec12* cells (MBY10-7C) to form heterozygous diploids. As shown in Fig. 1, the three lines showed different behaviors in diploids. STR1 × *sec12* kept the Ts$^+$ phenotype similar to the original STR1, indicating that this property of STR1 is dominant to the wild type. STR2 × *sec12* showed partial pappilar growth at 37°C and normal growth at 15°C. STR3 × *sec12* was Ts$^-$ and Cs$^+$ like the parent *sec12* cells. These indicate that the phenotypes of STR2 and STR3 are basically recessive to the wild type. The partial growth of STR2 × *sec12* at 37°C may imply codominance of the Ts$^+$ nature of STR2 but it was not potent enough to let all the diploid cells grow at 37°C.

Next, I performed tetrad analysis on these diploid cells. Unfortunately, the viability of the progeny spores was very low and only a few tetrads gave rise to four growing spores. Nevertheless, a large number of spores were examined for their growth phenotypes to enable random spore analysis. In all crosses, no more than half gave the original *sec12* phenotype (Ts$^-$) and the rest showed the revertant phenotype (Ts$^+$), indicating that the Ts$^+$ nature of these revertants was caused by a single nuclear mutation. I will call these mutated genes *RST1*^{DS}, *rst2*, and *rst3* hereafter, where *RST* stands for "revertant of sec twelve" and DS is for dominant suppressor. The Cs$^-$ phenotype of STR1 segregated from the suppression of *sec12* ts and therefore was not pursued further. For STR2 and STR3, Cs$^-$ was tightly linked to Ts$^+$, suggesting that these two phenotypes were due to the single mutations *rst2* and *rst3*.

STR1, STR2, and STR3 cells were also backcrossed to the *SEC12* cells (JRY9) and subjected to tetrad analysis. Again in random analysis, spore numbers of Ts$^+$:Ts$^-$ were 40:14 for STR1 × *SEC12*, 25:12 for STR2 × *SEC12*, and 41:14 for STR3 × *SEC12*. These ratios were close to 3:1 indicating random segregation of the *sec12 RSTX* spores (*RSTX* indicates the wild-type alleles where $X = 1, 2,$ or 3). This demonstrates that the *RST1*, *RST2*, and *RST3* loci are all distinct from the *SEC12* locus. In other words, they are all extragenic suppressors.

Whether *rst2* and *rst3* are allelic was tested as follows. The spores obtained from the above crosses were confirmed for their genotypes by another backcross and representative *rst2 sec12* and *rst3 sec12* haploids (Ts$^+$ Cs$^-$) with different mating types were chosen. The diploid cells generated by the mating of these two showed a Ts$^-$ Cs$^+$ phenotype. This indicated that the diploid strain was Rst$^+$ Sec12$^-$ and thus *rst2* and *rst3* defined distinct loci. I also noticed that the *rst3 sec12* cells give rise to Cs$^+$ Ts$^-$ cells at a high frequency. Genetic analysis on one of such second revertants indicated that this was due to another unlinked mutation. I have not investigated this mutation further. It should be also noted that *rst2* and *rst3* caused the Cs$^-$ phenotype in the *SEC12* background as well. *RST1*^{DS} showed no appreciable phenotype by itself.

Expression of Sec12p in the sec12 Revertant Cells—It is known that the *sec12-4* mutation has a ts defect in the guanine nucleotide exchange activity towards the Sar1 GTPase (*13*). It is also known that the elevated expression of the *sec12* gene suppresses this defect (*6*). When the *sec12* ts allele is introduced on a multicopy plasmid into the *sec12* mutant, the cells cease to show the ts growth. Such an increase in the expression of the mutant *sec12* product could be the cause of the revertant phenotypes. To test this possibility, I performed immunoblotting analysis using the anti-Sec12p antibody. As shown in Fig. 2, STR1, STR2, and STR3 cells all exhibited higher levels of Sec12p than the parent *sec12* or the wild-type cells. This is most evident with STR1. The band intensity of Sec12p is several-fold higher than the control. In the cases of STR2 and STR3, the increase of the amount of Sec12p is not as obvious as STR1, but the mobility in the SDS gel looks anomalous. We have shown that Sec12p is subject to complex modifications with oligosaccharides (*1, 14*). It receives both *N*- and *O*-linked modifications. The *N*-linked sugars are normally the high-mannose core plus $\alpha 1 \rightarrow 6$ mannosyl linkage, which is added in the early Golgi compartment during the ER-Golgi recycling (*14-16*). In the *rer1* mutants or when the retrieval signal of Sec12p is impaired, Sec12p is subject to further modifications, including $\alpha 1 \rightarrow 3$ mannosyl transfer that takes place in the medial compartment of the Golgi. The heterogeneous smeary pattern of Sec12p in the STR2 and STR3 cells is reminiscent of such abnormal modifica-

tions and will deserve further analysis. If the Sec12p molecule is in fact glycosylated heavily in the *rst2* and *rst3* mutants, this would suggest that the localization of Sec12p is affected by these mutations.

Is the increased expression in these STR cells specific for Sec12p? I examined the expression of Sar1p as well by immunoblotting. As shown in the lower panel of Fig. 2, there was no apparent difference in the Sar1p amount between STR and the parent *sec12* or the wild-type cells. Thus, the elevation of the cellular pool of Sec12p is not due to the general increase of the components involved in vesicle budding. Recently, Vahlensieck *et al.* (*17*) reported that the transcription of *SAR1* and some other *SEC* genes is not affected by conditions that may alter secretory activities. Whether the rise of Sec12p expression in the STR cells results from activated transcription or translation or from lowered degradation remains to be elucidated.

Protein Transport in the sec12 Revertant Cells—The suppression of the ts growth of *sec12* by the *RST* mutations suggests that they remedy the defect of vesicular transport as well. This was tested by looking at the processing of a vacuolar protein, carboxypeptidase Y (CPY). In the wild-type cells, newly synthesized CPY undergoes stepwise processing from the ER-precursor (p1) through the Golgi-precursor (p2) to the mature vacuolar form. Steady-state analysis of these species by immunoblotting detects only the mature form as the majority under normal conditions (Fig. 3). When the *sec12* mutant cells were incubated at 37°C for 2 h, the p1 form becomes detectable indicating that the ER-to-Golgi transport was blocked. Such accumulation of the p1 form was not detected when the STR1, STR2, and STR3 cells were cultured at 37°C. For the STR2 and STR3 cells, slight differences were noticeable in the mobility of the mature CPY: slower in STR2 and faster in

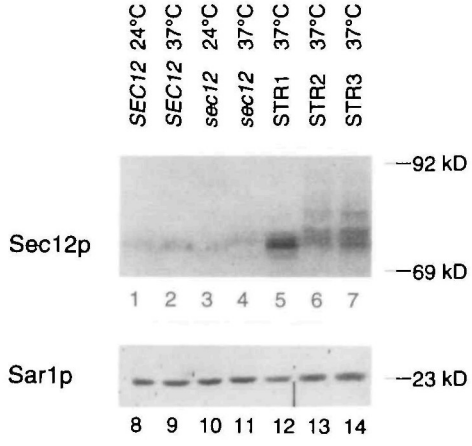

Fig. 2. **Immunoblotting analysis of Sec12p and Sar1p in STR cells.** *SEC12* (ANY21), *sec12* (MBY10-7A), and STR1, STR2, and STR3 cells were incubated for 2 h at the indicated temperatures and subjected to SDS-PAGE and immunoblotting using antibodies against Sec12p and Sar1p.

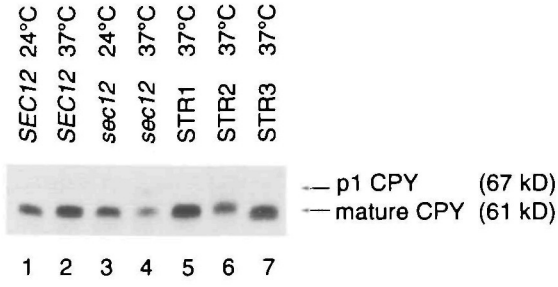

Fig. 3. **Immunoblotting analysis of carboxypeptidase Y (CPY) in STR cells.** The same cells as in Fig. 2 were analyzed with the anti-CPY antibody. p1 CPY, ER precursor form; mature CPY, mature vacuolar form.

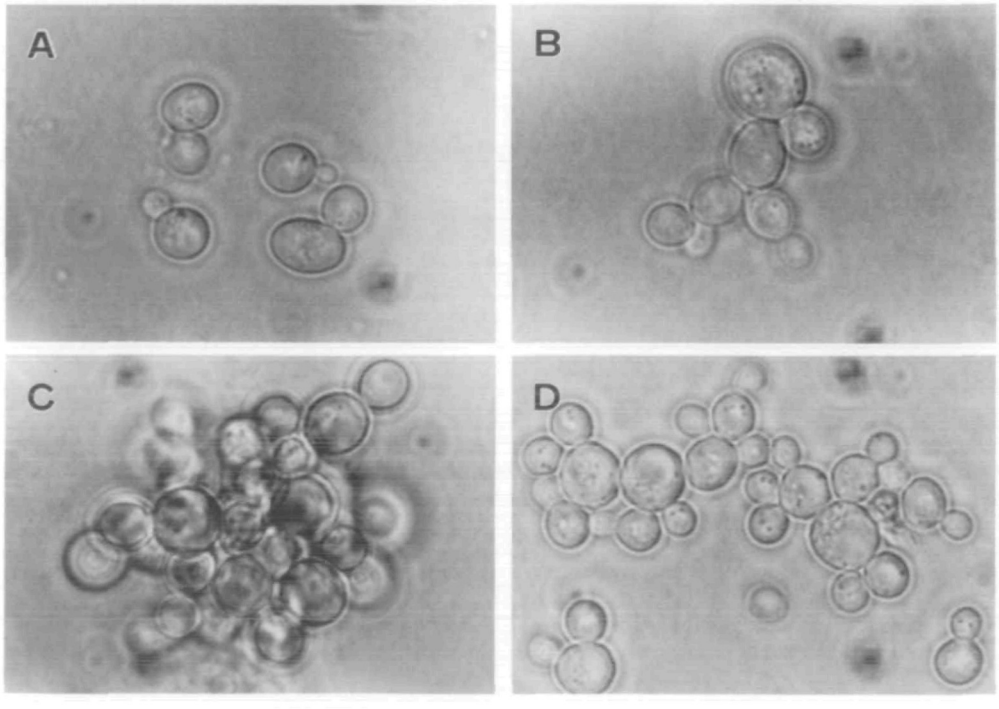

Fig. 4. **Cell morphology of STR cells.** *sec12* (MBY10-7A) (A), STR1 (B), STR2 (C), and STR3 (D) cells were grown in a liquid medium at 23 (*sec12*) or 37°C (STR) to the log phase and observed under a microscope. Phase contrast images are shown.

STR3. This could be due to different degrees of glycosylation on the CPY polypeptide but its significance is unclear at the moment.

Other Phenotype—STR2 and STR3 cells show morphological abnormality as well. As shown in Fig. 4, C and D, the cells do not separate very well after division and tend to aggregate in liquid culture. This was most prominent with STR2 and evident with STR3, but a similar tendency was also observed with STR1 occasionally (Fig. 4B). This phenotype may be consistent with the idea that these mutants are somehow affected in the oligo- or polysaccharide synthesis and thus implies involvement of the *RST* genes in secretory processes.

DISCUSSION

Genetics in yeast have been quite powerful to hunt for molecules that interact with a protein of interest. Around the *SEC12* gene product, *SAR1* has been identified as a multicopy suppressor of the *sec12* ts mutation (*5*) and at least three mutations, *sec13, sec16*, and *rer1*, have been shown to be synthetically lethal with the *sec12* mutation (*15, 18*). In this study, I attempted another way of identifying genetic interaction, namely, isolation of extragenic mutations that suppress *sec12*. Formally, suppressor mutations could occur in tRNA or other components of the protein synthesis system. The mutation point of *sec12-4* has been shown to be C to T transition at position 218, which causes Pro73 to Leu mutation. A missense suppressor mutation could lead to the reversion of the ts phenotype. However, it seems unlikely that such a translational mutation causes the pleiotropic phenotypes of the revertant cells I isolated.

To understand the molecular basis of *sec12* suppression, it will be important to clone the genes. Since the $RST1^{DS}$ allele is dominant to the wild type, we shall be able to clone it by constructing a genomic DNA library from the STR1 cells and screening for a clone that confers Ts^+ phenotype to the *sec12* cells. This attempt is now under way. The wild-type *RST2* and *RST3* genes should be easily cloned by complementation of the Cs^- phenotypes. Unfortunately, however, STR2, STR3, and their progeny cells were hardly transformed by DNA. The transformation efficiency by the conventional lithium method is miserably low. The protocol needs to be optimized for these mutant strains.

How could the temperature sensitivity of *sec12* be suppressed by these mutations? The most important piece of data is probably that the amount of Sec12p is increased in all of the mutant cells. As I mentioned above, the elevation of the mutant Sec12p expression can explain the suppression by itself. The problem is how these extragenic mutations cause the increase of Sec12p. One possibility may be the loss of regulation of the *SEC12* transcription. Transcriptional regulation has not been seriously considered for genes involved in secretion. The general belief is that the secretory processes are house-keeping and the genes must be constitutively expressed at least in yeast. The report by Vahlensieck *et al.* (*17*) also supports this idea. However, evidence is accumulating that regulation of gene expression is sometimes important for controlling secretion. For example, when the ER-to-Golgi transport is blocked by *sec* mutations, the transcription of the *KAR2* gene is elevated as a result of the accumulation of unfolded proteins (*19, 20*). An ER membrane protein, Ire1p, has been implicated in this regulation (*21-23*). It is also shown that upon blockade of vesicular transport along the secretory pathway, the transcription of ribosomal proteins and rRNA is repressed (*24*). These can be interpreted as a negative feedback to avoid the deleterious outcome of the block of secretion. Since Sec12p plays its role at the most upstream of the budding reaction from the ER, it is possible that elaborate regulatory mechanisms exist to control its expression. *RST1* is a candidate for such a regulator of expression. Its dominant mutation increases the expression of Sec12p but not that of Sar1p or CPY.

In the cases of *rst2* and *rst3*, it seems unlikely that simple alteration of gene expression is the cause of their phenotypes. The most interesting observation for me is that Sec12p appears to receive heterogeneous heavy glycosylation in these mutants. As mentioned above, such abnormal glycosylation could be an indication of mislocalization of Sec12p. Other observations, strange mobility of CPY in the SDS gel and a defect in cell wall separation, also point to anomaly in glycosylation. However, a simple deficiency in glycosylation cannot explain all of the phenotypes. What could be the primary defect that simultaneously affects the Sec12p amount, the Sec12p localization, general glycosylation and growth at low temperatures? Perhaps some unknown properties of the ER or the Golgi apparatus may be altered in the *rst2* and *rst3* mutant cells. If this is the case, I cannot rule out the possibility that the suppression of the *sec12* ts is not the direct consequence of the increase of Sec12p amount but is one of the pleiotropic results caused by such organellar alteration.

Apparently, information on the structure of these *RST* genes will give us more hints on their functions. We are now making great efforts along this line and will report elsewhere when the clones are obtained.

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