The Inactive Form of a Yeast Casein Kinase I Suppresses the Secretory Defect of the sec12 Mutant

IMPLICATION OF NEGATIVE REGULATION BY THE Hrr25 KINASE IN THE VESICLE BUDDING FROM THE ENDOPLASMIC RETICULUM*

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Sec12p is the guanine nucleotide exchange factor of Sar1p GTPase and functions at the very upstream in the vesicle budding reactions from the endoplasmic reticulum (ER). We previously identified three yeast loci, RST1, RST2, and RST3, whose mutations suppressed the temperature-sensitive growth of the sec12 (ts) mutant (Nakano, A. (1996) J. Biochem. (Tokyo) 120, 642–646). In the present study, we cloned the wild-type RST2 gene by complementation of the cold-sensitive phenotype of the rst2–1 mutant. RST2 turned out to be identical to HRR25, a gene encoding a dual-specificity casein kinase I in yeast. The rst2–1 mutation, which is now renamed hrr25–2, was due to the T176I amino acid replacement in the kinase domain. This mutation remedied not only the temperature-sensitive growth but also the defect of ER-to-Golgi protein transport of sec12. Immunoprecipitation of the hemagglutinin-tagged Hrr25p–2 protein and a subsequent protein kinase assay showed that the kinase activity of the mutant protein was markedly reduced. The overproduction of another kinase-minus mutant of Hrr25p (Hrr25p K38A) slightly suppressed the growth defect of sec12 as well. These observations suggest that the reduction of the kinase activity in the mutant protein is important for the suppression of sec12. We propose that Hrr25p negatively regulates the vesicle budding from the ER.

The secretory pathway begins from the ER.1 By genetic approaches with yeast Saccharomyces cerevisiae, many secretory genes have been identified in the whole pathway, and more than 20 genes are now known to function in the transport from the ER to the Golgi apparatus. Among these genes, SEC12 and SAR1 are believed to play pivotal roles in the earliest step, that is the formation of transport vesicles from the ER. SAR1 encodes a 21-kDa GTPase (Sar1p) (1), which functions as a molecular switch to recruit a coat protein complex, COPII, onto the ER membrane (2); SEC12 codes for a 70-kDa integral membrane protein (Sec12p) in the ER and acts as the guanine nucleotide exchange factor (GEF) toward Sar1p, which converts Sar1p from the inactive GDP form to the active GTP form (3). Sar1p-GTP promotes the assembly of COPII (Sar1p, Sec13p, Sec31p, and Sec23p/Sec24p) (2), budding, formation, and release of vesicles (2, 4). Thus, Sec12p is the most upstream player in the vesicle budding from the ER as far as we know. However, little is known as to when and how the GEF activity of Sec12p is triggered in this earliest event of vesicle budding. The regulation of Sec12p function may be a key issue for understanding the mechanisms of cargo and resident selection in the vesicle budding event.

We recently identified three genetic loci, RST1, RST2, and RST3, whose mutations suppressed the temperature-sensitive (ts) growth defect of the sec12 (ts) mutant (5). These genes were expected to be the candidates of Sec12p regulators. RST1–1 was a dominant mutation and caused elevated expression of Sec12p. rst2 and rst3 were recessive and gave pleiotropic phenotypes including slow growth at low temperature, aggregation of cells, and heterogeneous glycosylation of Sec12p. In this study, we extended characterization of the rst2–1 mutant and cloned the wild-type RST2 gene by complementation.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions—The yeast strains used in this study were MBY10–7A (sec12–2 ura3–52 leu2–3, 112 trp1–289 his3 his4 suc gal2 MA TA) (6), MBY10–7C (sec12–4 ura3–52 leu2–3, 112 trp1–289 his3 his4 suc gal2 MA TA) (6), ANY21 (ura3–52 leu2–3, 112 trp1–289 his3 his4 suc gal2 MA TA) (6), STR2 (sec12–4 rst2–1 ura3–52 leu2–3, 112 trp1–289 his3 his4 suc gal2 MA TA) (5), and AMY7–4B (2μ HRR25 URA3; hr r25: HIS3 ura3–52 lys2–801 ade2–101 trp1-D63 his3-D200 leu2-D1 MA TA) (this study). The cells were grown at 37 °C (for STR2), 30 °C or 23 °C (for MBY10–7A and MBY10–7C) in YP medium (2% (w/v) polypeptide (Nihon Pharmaceutical Co. Ltd., Tokyo, Japan), 1% (w/v) yeast extract (Difco Laboratories, Inc., Detroit, MI), 2% (w/v) glucose (Difco Laboratories)) containing 0.5% (w/v) casamino acids (Difco Laboratories) and 0.07% (w/v) yeast nitrogen base without amino acids (Difco Laboratories). 0.5% (w/v) adenine, 0.01% (w/v) uracil, and 0.005% (w/v) biotin were added. A 10-fold higher concentration of uracil was used for the strain RST2 (r st2–1 sec12–14), which shows a C a T phenotype, which was transformed with a genomic library (10) constructed on YEp13, which contains the LEU2 gene as the selectable marker. Electroporation was used to obtain high transformation efficiency (see below). Transformants were plated in 5–6 ml of regeneration top agar containing 1× MVD, 1 M sorbitol, 2.5% agar, and appropriate supplements and incubated at 24 °C. After 5–6 days, large colonies were picked up, streaked, and plated on regeneration agar plates and incubated at 24 °C.
and further incubated at 15 and 37 °C. Among eight candidates we obtained, one clone (named P2–3) reproducibly conferred Cs'–Tσ-growth to the STR2 cells. This plasmid was recovered from the yeast transformant. After the confirmation of the phenotypes by retransformation, the genomic insert was subcloned and subjected to further complementation tests.

Transformation by Electroporation—From a saturation culture in 50 ml of YDP, STR2 cells were harvested and washed twice with sterile distilled water. The cells were resuspended in 20 ml of 0.1 M Tris-HCl, pH 9.4, and 100 µl of 1 × DTT. After incubation at 30 °C for 10 min, the cells were centrifuged at 1800 × g for 5 min and resuspended in the speriplasting buffer (0.67% yeast nitrogen base, 1 M sorbitol, and 0.5% glucose). Zymolyase-100T (Seikagaku Corp.) (5 mg) was added to the cell suspension, and incubation was continued for 10–20 min at 30 °C. The Zymolyase-treated samples were overlaid on 25 ml of 1.4% sorbitol solution and centrifuged at 1800 × g. Spheroplasted pellets were resuspended in 0.5 ml of 1 M sorbitol and kept on ice until use for electroporation.

For electroporation, 4 µg of DNA (up to 10 µl) was added to a 100-µl aliquot of the spheroplast suspension. The samples were mixed gently and put on ice 5 min prior to electroporation, and then transferred to a cold sterile cuvette (0.4 cm) and subjected to electroporation (Bio-Rad Gene Pulser®; pulse at 1.5 kV, 25 microfarads, 200 watts for 3 s). Immediately after electroporation, 100 µl of 1 × sorbitol solution was added and the samples were spread on MVD plates (with adequate supplements) containing 1 M sorbitol.

Introduction of the Influenza Hemagglutinin (HA) Tag into HRR25—The AflIII-AflIII fragment of HRR25 was amplified with an NheI site created near the 5′-terminus of the ORF by PCR (primer sequences: 5′-TCTAGGACTTAAAGCTAAGCTTGAAGGAATTT-3′ and 5′-CTTCGCTTAAGATCTGACAGACG-3′). The resulting NheI site-containing fragment was used to replace the original HRR25-AflIII-AflIII fragment. The DNA cassette encoding three tandem repeats of the HA epitope was excised from pYTI1 by Nhel digestion (11), and inserted into the Nhel site of the above construction. The resulting plasmid was named pAM5–2 (2 µg). HRR25 TRP1. The expression of 3HA-HRR25 was confirmed by immunoblotting with the monoclonal anti-HA antibody 16B12 (Berkeley Antibody). A plasmid harboring 3HA-rst2–4, PAM6–2 (2 µg), and pAM5–2 (2 µg) was constructed from pAM1–5 (5 µg) and 3HA-rst2–1 TRP1) by substituting the AflIII-AflIII fragment of pAM5–2 for that of pAM1–5. pAM5–5 is the plasmid containing the mutant allele rst2–1, which was obtained by the allele recovery method.

The HRR25 expression plasmid, pAM4–1 (2 µg) GAL1 promoter 3HA-HRR25 URAS3), was constructed as follows. A HindIII site was created just before the initiation codon of HRR25 by the BamHI-HindIII fragment containing 3HA-HRR25 was subcloned into pYES2 (Invitrogen), Leek, The Netherlands), which is a 2µ-based multicopy plasmid carrying the GAL1 promoter and a selectable URA3 marker.

Site-directed Mutagenesis in 3HA-HRR25—The K38A mutation in 3HA-HRR25 was created by PCR with the following primers: for a N-terminal fragment, 5′-GGTCGCCGAAAGAGCTTAGTGTGGTG-3′ (hrr25 XHOI) and 5′-ATCACTGTACCTGCGGCCG-3′ (hrr25 NCOI) and 5′-ATGACGATCCCTGCGGCCG-3′ (hrr25 NCOI) were used for C-ter and HI-I digestion (11), and inserted into the NheI site of the above construction. The resulting plasmid was named pAM6–2 (2 µg). HRR25. The BamHI-HindIII fragment containing 3HA-HRR25 was subcloned into pYES2 (Invitrogen), Leek, The Netherlands), which is a 2µ-based multicopy plasmid carrying the GAL1 promoter and a selectable URA3 marker.

Sec12 Suppresses the Secretory Defect of the sec12 Mutation—To examine whether rst2–1 suppresses not only the temperature-sensitive growth defect but also the secretory defect of the sec12–4 mutation, we performed a pulse-chase and immunoprecipitation experiment with the anti-carboxypeptidase Y (CPY) antibody (Fig. 1, upper panel). In the wild-type cells, newly synthesized CPY undergoes stepwise processing from the 67-kDa ER precursor (p1) through the 69-kDa Golgi precursor (p2) to the 61-kDa mature vacuolar form (m). When the sec12 mutant cells were pulse-labeled for 4 min and chased for 2 h at 37 °C, the p1 form accumulated, indicating that the ER-to-Golgi transport was blocked. Such accumulation of the p1 form was not detected when the sec12–4 rst2–1 double mutant cells were incubated at 37 °C. Normal modification and processing from p1 through p2 to m forms was observed, although the rate was a little slower than that of wild-type cells.

We also performed a pulse-chase experiment on a glycosylphosphatidylinositol-anchored plasma-membrane protein, Gas1p (14, 15) (Fig. 1, lower panel). Like the case of CPY, the immature form (i) of Gas1p accumulated in the sec12 mutant cells after 30-min chase at 37 °C. In contrast, Gas1p was processed to the mature form (m) in the sec12–4 rst2–1 double mutant cells at the restrictive temperature for sec12, 37 °C. These data indicate that the rst2 mutation remedies the secretory defect of the sec12 mutation.

Overproduction of the Sec12 ts protein by the introduction of the mutant gene on a multicopy plasmid (2 µg) allows growth of the sec12 ts strain at 37 °C (Fig. 2A; see also Ref. 16). Even a slight increase of the gene dosage by the introduction of the
single-copy plasmid (CEN) of sec12 could suppress the ts growth to some extent. We performed immunoblotting analysis using the anti-Sec12p antibody to examine whether the level of Sec12–4p increased in the sec12–4 rst2–1 cells to the extent that was able to suppress the sec12 mutant phenotype. As shown in Fig. 2B, the amount of Sec12–4p was slightly increased in the sec12–4 rst2–1 cells as compared with that in the sec12–4 RST2 cells (lanes 3 and 4 versus lanes 1 and 2). Careful quantification of the results of three independent experiments with the amount of Pgk1p as an internal standard indicated that the amount of Sec12–4p was 2–3 times larger in sec12–4 rst2–1 than in sec12–4 RST2. The extent of the increase was almost the same as the case where an extra copy of sec12–4 was supplied by the introduction of a single-copy (CEN) plasmid.
Casein Kinase I Regulates Vesicle Budding from the ER

Fig. 4. The mutation point of hrr25–2. A, illustration of the HRR25 gene product (Hrr25p). T176I is the mutation point of hrr25–2 (≡ rst2–1). Note that Hrr25p contains a region rich in proline and glutamine residues at the C terminus (Pro/Gln-rich). B, comparison of amino acid sequences in the kinase homology region between Hrr25p and other yeast CKIs. Black boxes represent the residues identical among the four CKIs, and the asterisk indicates the hrr25–2 mutation.

Cloning of the Gene That Complements the rst2 Mutation: HRR25—For the cloning of the RST2 gene, STR2, the original cold-sensitive rst2–1 sec12–4 mutant strain, was transformed with a yeast genomic DNA library constructed on the multicopy plasmid YEp13 (10). DNA clones that rescued the cold-sensitive growth were selected. One clone named P2–3 showed good complementation and was analyzed further. The 6.7-kb insert of P2–3 contained two complete ORFs, HRR25 and TPK2/PKA3 (see Fig. 3A). To localize the complementation activity of rst2–1 in this insert, deletion analysis was performed. Various fragments from P2–3 were subcloned into a multicopy plasmid, pAM2–3, or a single-copy CEN plasmid, pRS314, and introduced into the rst2–1 sec12–4 mutant (STR2). Transformants were tested for growth at the restrictive temperature for rst2–1, 15 °C. As shown in Fig. 3A, DNA fragments always complemented the rst2 mutant when they contained HRR25. The presence or absence of TPK2 did not correlate with the complementation activity. The same results were obtained for both the multicopy and single-copy plasmids. Thus, it is the HRR25 gene that complemented rst2–1. STR2 cells show morphological abnormality as well (5). The cells do not separate very well after division and tend to aggregate either in liquid or on plate culture. The DNA fragment containing HRR25 not only resumed the growth defect of STR2 at 15 and 23 °C (Fig. 3B), it also remedied this aggregation phenotype (Fig. 3C).

To confirm that HRR25 is the authentic RST2 gene, the fragment containing HRR25 with the LEU2 marker was integrated at the HRR25 locus in the sec12–4 mutant, MBA10–7C. The integrant was mated with STR2 which was transformed by pAM2–326, a URA3-marker multicopy plasmid containing HRR25 derived from pYO326. This plasmid complemented rst2–1 and dramatically improved the mating and sporulation efficiency of the mutant. The diploid cells were sporulated, and the progeny haploid cells were plated on MVD (complete supplement) containing 5-fluoroorotic acid (FOA) at 27 °C to remove pAM2–326. Among 160 spores analyzed, 67 spores showed Leu−Cς− phenotype, 90 spores were Leu− Cς−1, 1 was Leu− Cς−2, and 2 were Leu− Cς−. This result indicated that the LEU2 marker was tightly linked to the Cς− phenotype, namely HRR25 was linked to the rst2–1 locus. Therefore, we concluded that RST2 is identical to HRR25. Finally, we renamed our mutant allele (rst2–1) of HRR25, hrr25–2.

hrr25–2 (rst2–1) Is a Mutant with a Reduced Kinase Activity—HRR25 encodes a dual-specificity casein kinase I (CKI) (17). We isolated the mutant hrr25–2 gene by the allele recovery method and determined that it contained a mutation of the C527T replacement in the nucleotide sequence, which caused T176I mutation in the amino acid sequence (Fig. 4A). A comparison of the amino acid sequence of Hrr25p with other yeast CKIs is shown in Fig. 4B. The T176I mutation is in the region conserved in all members of the yeast CKI family. To examine whether hrr25–2 is a kinase-minus (low kinase activity) or constitutively active mutant, we carried out an in vitro kinase assay for the Hrr25 protein from the wild-type and mutant cells.
the immunoprecipitation kinase assay (Fig. 5, lane 7), in fact, we could not detect any kinase activity of 3HA-Hrr25p K38A. As shown in Fig. 6A, the overproduction of Hrr25p K38A slightly suppressed the growth defect of sec12–4 at 35 °C. On the other hand, it inhibited the growth of sec23–1 and sar1–2 at 30 and 33 °C, respectively, and that of the wild-type slightly at 35 °C.

Reduction of the Kinase Activity but Not Complete Loss of Hrr25p Is Important for the sec12–4 Suppression—A HRR25 disruption was generated by inserting the 1.8-kb BamHI fragment of HIS3 from pJJ215 into the AphII-NdeI sites of HRR25. This disrupted gene was introduced on an integration vector into the wild-type diploid, YPH501. The resulting heterozygous diploid was sporulated and subjected to tetrad dissection. Fast and slow growing spores segregated 2:2. All the slow growth spores showed His+ phenotype. The correct integration at the HRR25 locus was confirmed by Southern blotting (data not shown). This indicates that these slow growing spores were the Δhrr25 mutant. This observation is consistent with the results of Hoekstra et al. (17), i.e. HRR25 is not essential but very important for cell viability. Δhrr25 showed extremely slow growth at low (15 °C) and high (37 °C) temperatures.

If the suppression of sec12–4 by hrr25–2 was due to the reduction of the Hrr25p kinase activity, the disruption of HRR25 would also suppress the sec12–4 growth defect. To test this, the Shrr25 mutant cells harboring pAM2–326 (2μ HRR25 URA3) were mated with the sec12–4 mutant (MBY10–7C). This diploid was sporulated, and tetrads were dissected to obtain segregants of the genotype, Δhrr25 sec12–4. The spores showed a 2:2 segregation pattern regarding the sec12–4 phenotype as marked by growth Ts+ on MCD (−Ura) plates. If these spores were cultured on FOA-containing plates at 15 °C, they also showed a 2:2 segregation pattern, because Δhrr25 cells were extremely slow in growth at high temperature (15 °C). We selected segregants that are Ts+ on MCD (−Ura) and Cs− on FOA plates, which have the genotype of Δhrr25 sec12–4. The segregants were grown on FOA plates at 27 °C to lose the HRR25 plasmid and examined for growth at high temperatures. As shown in Table I, they were all unable to grow on YPD plates at either 35 or 37 °C. This result indicates that Shrr25 cannot suppress the sec12 mutation and suggests that hrr25–2 is not a null mutation in terms of the sec12 suppression. We also constructed double mutants of hrr25–2 with sec13–1, sec23–1, and ret1–1, but no clear suppression was observed.

To further analyze the relationship between the kinase activity and the sec12 suppression, the two mutant alleles of HRR25, hrr25 K38A and hrr25 T176I, were expressed in the Δhrr25 sec12–4 double mutant cells. As shown in Fig. 6B, Δhrr25 sec12–4 hardly grew at 35 °C. The mutant cells expressing HRR25 did not grow at 35 °C, either, due to the sec12–4 mutation. However, the expression of hrr25 K38A or hrr25 T176I suppressed the growth defect at 35 °C at least to some extent.

**DISCUSSION**

In this paper, we have shown evidence for the first time that Hrr25p, a yeast CKI, is involved in vesicle budding from the endoplasmic reticulum.

**Family of Yeast CKI**—CKI is an expanding family of kinases, which have been proposed to play a variety of roles in many cellular processes. Among five defined isoforms of mammalian CKI (18–20), for example, the α isoform has been implicated in the regulation of secretion (21).

The yeast *S. cerevisiae* possesses four genes that encode CKI: *YCK1*, *YCK2*, *YCK3*, and *HRR25*. The redundant *YCK1* and *YCK2* genes are required for cell viability (22) and morphogenesis (23). Recently, several reports have suggested that *YCK1*,
YCK2, and YCK3 are involved in vesicular transport through the studies of their genetic interactions. For example, suppressor mutations of the yck1Δ yck2–2 mutant (yck2Δ) define four subunits of a novel clathrin AP-like complex, AP-3 (24). The yck2Δ mutant shows a strong synthetic growth defect with chc1-ts (24), and exhibits ts phosphorylation, ubiquitination, and endocytosis of the α-factor receptor (25). YCK1, YCK2, and YCK3 suppress the defect of the deletion of GCS1, a yeast ARF GTPase-activating protein gene (26, 27), in cell proliferation from the stationary phase (28). HRR25 forms an essential gene pair with YCK3 but cannot suppress the Δgcs1 mutant. These relationships are illustrated in Fig. 7. In contrast to such implications of YCK1, YCK2, and YCK3 in vesicular traffic, HRR25 was originally proposed to carry out other important function(s). The HRR25 gene was identified by a mutation that conferred sensitivity to the expression of HO. HO is a gene coding for a 65-kDa endonuclease, which performs site-specific cleavage of double-stranded DNA, and is essential for the initiation of mating-type interconversion. Using a yeast strain harboring a galactose-inducible HO gene, Hoekstra et al. (17) isolated mutants that were unable to grow on galactose-containing medium. One of the mutants, hrr25–1, showed sensitivity to continuous expression of the HO double-strand endonuclease, to methylmethanesulfonate, and to x-ray irradiation. The hrr25–1 mutant cells not only had a defect in DNA double-strand break repair, but also showed poor sporulation, very slow growth, and cell cycle delay in G2 (17).

Yck1p, Yck2p, and Yck3p have a motif of prenylation (GCC) in their C termini, and in fact Yck2p is tightly associated with the plasma membrane (29). Hrr25p does not have the prenylation motif, but was also found exclusively in membrane fractions in differential centrifugation analysis; HA-tagged Hrr25p cofractionated with plasma membrane and nuclei (29). DeMaggio et al. (30) predicted that Hrr25p is a multipotential protein kinase, generating phosphothreonine and phospho-

![Image](320x304 to 542x443)

Fig. 7. Genetic interactions between yeast CKIs and components involved in vesicular transport. See “Discussion” for details. AP-3, adaptor protein complex 3; GCS1, a gene encoding GTPase-activating protein of ARF; CHC1, the gene encoding clathrin heavy chain.

![Image](152x535 to 452x729)

Fig. 6. Overexpression of the dominantly acting kinase-minus mutant of Hrr25p (3HA-Hrr25p K38A) in the sec12–4 mutant and Δhrr25 sec12–4 double mutant cells. A, multicopy (2μ), GAL1-promoter-driven HA-tagged hrr25 K38A (K38A), or vector alone (vector) was expressed in sec12–4 (MBY10–7A), sec23–1 (MBY8–20C), sar1–2 (TOY224), and wild-type (ANY21) cells. Overnight liquid cultures of these transformants were diluted from 1 × 10^7 to 1 × 10^6 cells/ml by 10-fold serial dilution (A, left to right), and 5 μl each of diluted samples were spotted on MCGS plates. The cells were cultured for 8 days at the indicated temperatures. B, the Δhrr25 sec12–4 mutant cells harboring HRR25 (pAM2–326: URA3-marked multicopy plasmid) were transformed with multicopy, own-promoter-driven hrr25–2 (T176I), HA-tagged HRR25 (HRR25), hrr25 K38A (K38A), or vector alone. The transformants were cultured for 6 days at 26 °C on MCD plates (complete supplements containing 5-fluoroorotic acid to remove pAM2–326, and then restreaked on MCD (minus tryptophan) plates and cultured for 14 days at 26 and 35 °C.

| Genotype          | Growth at 35 and 37 °C |
|-------------------|------------------------|
| Δhrr25 sec12–4    | 29                     |
| Δhrr25 SEC12      | 33                     |
| HRR25 sec12–4     | 34                     |
| HRR25 SEC12       | 0                      |

![Image](152x535 to 452x729)
cells grow slowly; their doubling time is approximately 4–5 times longer than that of the wild-type. However, the presumed kinetics of CPY transport is as fast as in the wild-type. Thus, besides the functions in DNA damage repair and cell cycle control, Hrr25p must play some role in the secretory transport pathway.

hrr25–2 harbors a mutation that causes the amino acid replacement T176I. This residue is conserved among the four yeast CKIs and the mutant Hrr25p T176I protein with an HA tag showed little kinase activity. We constructed another mutant allele of HRR25 that causes the K38A replacement. This residue is also conserved in the kinase domain, and the mutant protein was shown to have lost the kinase activity. The overexpression of Hrr25p K38A also suppressed the ts growth of sec12–4, although weakly. These results suggest that it is the decrease of the kinase activity that is important for Hrr25p to suppress sec12–4. However, the deletion of HRR25, which is not a lethal event, did not suppress sec12–4.

Two reasons can be considered to explain the difference between the loss-of-function-type missense mutations (T176I and K38A) and the null mutation. The hrr25 deletion is not lethal, but the disruptive cells are quite sick. They grow very poorly at 30 °C and are almost invisible at high and low temperatures. Perhaps the null mutant cells have so many problems to sustain growth, and even though the loss of activity had a remedial effect on sec12–4, other lesions may have concealed such suppression. The alternative, more intriguing possibility is that the presence of the mutant protein is required for the suppression. The observation that the overproduction of Hrr25p T176I or K38A allowed the growth of the sec12–4 suppressor mutant implies again the important role of Sec12p in vesicle budding from the ER.

Although the interaction between the active kinase and substrates may be transient, a kinase-inactive mutant may form a stable protein–protein complex. In mitogen-activated protein kinase pathways, some substrates have been identified by two-hybrid screening with such a kinase-minus mutant as a bait (32–34). It is possible that Hrr25p T176I or K38A binds to its substrate(s) very tightly and, by doing so, releases the budding block of sec12–4. There are some differences between Hrr25p T176I and K38A. For example, the overexpression of Hrr25p K38A suppresses ∆hrr25 sec12–4 better than that of Hrr25p T176I, even though K38A appears to be more severely impaired in the kinase activity. This could be explained by the positions of mutations; K38A is in the ATP-binding site and T176I in a putative substrate recognition site.

**Target of Hrr25p in Vesicular Traffic—**Sec12p is not phosphorylated under a normal condition of vegetative growth as far as we examined by *in vivo* labeling experiments, and is thus unlikely to be a substrate of Hrr25p. This led us to postulate that the target of the Hrr25p in vesicular transport is a negative regulator of the Sec12p function. It could be directly regulating the function of Sec12p or might transduce a signal to control vesicle budding reactions. Then, what is this putative regulator? Sec31p, one of the COPII components, was a good candidate because it had been shown to be a phosphoprotein and the phosphatase treatment of Sec31p/Sec31p complex inhibited vesicle budding (36). However, Sec31p was still phosphorylated in the hrr25–2 mutant cells, and the purified Sec13p/Sec11p complex was not subject to phosphorylation by 3HA-Hrr25p in our *in vitro* kinase assay. At the moment, we have not yet been able to identify *in vivo* or *in vitro* substrates of Hrr25p as candidates of the regulator of the Sec12p function. Considering the possibility that the titration of a regulator of Sec12p by the kinase-minus Hrr25p mutant (K38A or T176I) is the cause of the sec12 suppression, this regulator might not necessarily be a substrate of the Hrr25p kinase.