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Identification of *SEC12*, *SED4*, Truncated *SEC16*, and *EKS1/HRD3* as Multicopy Suppressors of ts Mutants of Sar1 GTPase[1]

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The yeast *SAR1* gene encodes a low-molecular-weight GTPase which is essential for the formation of transport vesicles from the endoplasmic reticulum (ER). To understand how the Sar1p function is regulated in its GTPase cycle, we searched for multicopy suppressors of *sar1* temperature-sensitive mutants and identified *SEC12*, *SED4*, truncated *SEC16*, and *EKS1*. *EKS1* turns out to be identical to *HRD3*, which was independently isolated as a gene implicated in the degradation of an HMG-CoA reductase isozyme, Hmg2p. In this paper, we show that the product of *EKS1/HRD3* is a type-I transmembrane glycoprotein and resides in the ER. The *eks1/hrd3* disrupted cells are normal in growth and transport of cargo proteins, but missecrete BiP (Kar2p). The overexpression of *EKS1/HRD3*, which stabilizes Hmg2p, did not affect the stability of wild-type or mutant Sar1p or any early Sec proteins we examined. These results suggest that the role of Eks1p/Hrd3p is not involved in the ER protein degradation in general but rather required for the maintenance of the ER membrane functions. The novel genetic interactions unveiled between *SAR1*, *SEC12*, *SEC16*, and *SED4* will provide useful information as to how the complex machinery of vesicle budding operates.

Key words: COPII vesicle, ER protein degradation, *Saccharomyces cerevisiae*, *SAR1*, vesicular transport.

Intracellular transport between membrane-bounded organelles in the secretory pathway is mediated by vesicles. In this vesicular traffic, a huge number of low-molecular-weight GTPases have been shown to function at a variety of steps. Among them, the Sar/Arf family of GTPases are required for the budding and formation of transport vesicles from donor compartments, whereas Rab/Ypt GTPases are involved in the targeting and fusion of vesicles with acceptor compartments (*1, 2*).

Sar1 GTPase (Sar1p), the product of the yeast *SAR1* gene, is a key player in the initial events of transport from the endoplasmic reticulum (ER) to the Golgi apparatus. *SAR1*, originally isolated as a multicopy suppressor of the yeast *sec12* ts mutant (*3*), has been shown to be essential for the formation of transport vesicles from the ER in yeast (*4-6*) in mammalian cells (*7*), and probably in plant cells (*8, 9*). The vesicle budding reaction begins with the conversion of Sar1p-GDP to Sar1p-GTP by the guanine-nucleotide exchange factor (GEF), Sec12p, which is an integral membrane glycoprotein in the ER (*5, 10*). In the GTP-bound active state, Sar1p recruits coat proteins (COPII; Sec23p/Sec24p and Sec13p/Sec31p complexes) onto the ER membrane and promotes budding of transport vesicles (*11*). Hydrolysis of GTP by Sar1p is triggered by the GTPase-activating protein (GAP), Sec23p, after vesicle formation is completed (*6, 12*). Once GTP is hydrolyzed on the vesicles, Sar1p is released into the cytosol, which leads to the disassembly of the coats (*11*). The released GDP-bound Sar1p recycles to the ER. Sec16p is also implicated in this step by its tight interaction with Sar1p and other COPII components (*3, 13-16*).

To further understand the role of the GTPase cycle of Sar1p, we have isolated and characterized three *sar1* temperature-sensitive (ts) mutants (*17-19*). Two ts alleles, *sar1*D32G and *sar1*N132I, have mutations in the GTP binding consensus sequences, and the third one, *sar1*E112K, has a replacement at a glutamate residue that is highly conserved in all members of the *SAR1* family. These ts mutants all show a defect in transport from the ER to the Golgi apparatus and accumulate ER membranes at the restrictive temperature (*18*). Purified Sar1pE112K and Sar1pD32G preferentially bind GDP to GTP and are thus regarded as GDP-form mutants (*19*). Here we report the identification of four genes, *SEC12*, *SED4*, truncated *SEC16*, and *EKS1/HRD3*, as multicopy suppressors of

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Abbreviations: BiP, binding protein; ER, endoplasmic reticulum; GAP, GTPase-activating protein; GEF, guanine-nucleotide exchange factor; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; ts, temperature sensitive; ORF, open reading frame.

sar1 ts mutants. The roles of these suppressor genes in regulation of the Sar1p function will be discussed.

MATERIALS AND METHODS

Strains and Culture Conditions—The *Saccharomyces cerevisiae* strains used in this study are listed in Table I. Yeast cells were grown in YPD medium [1% yeast extract (Difco Laboratories), 2% polypeptone (Nihon Seiyaku), and 2% glucose] or in MVD [0.67% yeast nitrogen base without amino acids (Difco Laboratories) and 2% glucose] supplemented appropriately. MCD medium is MVD containing 0.5% casamino acids (Difco Laboratories).

Plasmids—As the subclones of *SEC12* and *SAR1*, the 6.4-kb *Xho*I-*Xba*I fragment of pSEC1230 (*10*) and the 1.3-kb *Hin*dIII-*Sma*I fragment of pANY2-7 (*3*) were inserted into pYO325 (*20*), and the resulting plasmids were named pYO12 and pYOSAR. The *Acc*III-*Sal*I fragment of *SED4* was cloned from a λ phage (provided by S. Tanaka and K. Isono of Kobe University) containing the corresponding region of the yeast chromosome III (*21*) and subcloned into pYO325 to obtain pANY4-2.

pSEC1310 and pSEC1610 are the original genomic clones containing *SEC13* and *SEC16* genes, respectively, which were isolated by M. Bernstein (University of California, Berkeley). Various subclones were constructed to determine which part of these plasmids contained the suppressor activity toward *sar1* ts mutants. The 3.0-kb *Hin*dIII-*Nsp*V fragment containing the open reading frame YLR207w (*EKS1/HRD3*) was inserted into pSQ326 (*20*) and pYO325 to yield pYS3 and pYO112KS-1, respectively.

Disruption of the EKS1 Gene—The *eks1* disruptant cells were constructed as follows. The 3.2-kb *Hin*dIII-*Xba*I fragment of pSEC1310 was blunted with T4 polymerase and inserted into the *Not*I/*Hin*cII sites of pBluescriptII SK$^+$ (pYS1-6). The 0.9-kb *Sma*I-*Bgl*II fragment of pJJ281 (*22*) containing the *TRP1* gene was inserted into the *Nhe*I/*Bam*HI sites of pYS1-6 (pYS2-2B). The 3.4-kb *Sac*I-*Xho*I fragment of pYS2-2B was excised and used to transform wild-type diploid cells (YPH501). The replacement of the *EKS1* gene by its disrupted copy was confirmed by Southern blotting. The obtained heterozygous cells were sporulated and subjected to tetrad dissection. One of the Trp$^+$ progenies was selected and named YSY37.

Epitope Tagging of EKS1 and SED4—The epitope-tagged *EKS1-3HA* was constructed as follows. An *Xba*I site was created in pYS1-6 at the amino acid positions 61 and 62 of Eks1p by site-directed mutagenesis using the oligonucleotide 5′-GTGTTAATAAGGGCTGATTCTAGAGCGGACCCTTGGCCTG-3′. The resulting plasmid was named pYS1-6XB. The *Nhe*I-*Nhe*I fragment from pYT11 (*23*), encoding three tandem copies of the hemagglutinin (HA) epitope, was inserted into this *Xba*I site of pYS1-6XB to produce pYS1-HAB. The 3.5-kb *Sac*I-*Xho*I fragment of pYS1-HAB containing *EKS1-3HA* was subcloned into pRS316 (*24*) and pSQ326 to give pYS14 and pYS15, respectively.

SED4 was also epitope-tagged as follows. To insert the 3HA epitope before the C-terminal HDEL sequence, an *Nhe*I site was introduced by site-directed mutagenesis using the oligonucleotide 5′-GGAACCGTAAACTACGCTAGCCTTCATGACGAATTGTGA-3′. The *Nhe*I-*Nhe*I fragment of pYT11 was inserted into this *Nhe*I site of *SED4* and subcloned in pRS314 (*24*) to produce pYS28-E52.

All the constructions were confirmed by DNA sequencing.

Antibodies—Rabbit antibodies against BiP (Kar2p), Sec16p, and Sec24p were provided by M. Rose (Massachusetts Institute of Technology), C. Kaiser (Massachusetts Institute of Technology), and R. Schekman (University of California, Berkeley), respectively. Anti-Sec12p and anti-Sar1p antibodies were described previously (*3, 10*). Mouse monoclonal anti-HA antibodies (16B12 and 12CA5) were purchased from Berkeley Antibody Company (Richmond, CA) and Boehringer Mannheim Yamanouchi (Tokyo), respectively.

Immunofluorescence Microscopy of Eks1-3HAp—The intracellular localization of Eks1p was examined by indirect immunofluorescence microscopy as previously described (*25, 26*). The *eks1* disruptant cells carrying *EKS1-3HA* on a single-copy plasmid (YSY37/pYS14) were grown to a logarithmic stage, fixed, and spheroplasted. To double-

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

Strain	Genotype	Reference
YPH501	*MATα/MAT***a** *ura3/ura3 lys2/lys2 ade2/ade2 trp1/trp1 his3/his3 leu2/leu2*	a
MBY10-7A	*MAT***a** *sec12-4 ura3 leu2 trp1 his3 his4*	b
MBY3-15A	*MATα sec13-1 ura3 leu2 his3*	b
MBY4-1A	*MAT***a** *sec16-2 ura3 trp1 his3*	b
MBY5-2A	*MAT***a** *sec20-1 ura3 trp1*	b
MBY6-4D	*MATα sec21-1 ura3 leu2 trp1 his3/4*	b
MBY13-2D	*MAT***a** *sec22-3 ura3 trp1*	b
MBY8-20C	*MATα sec23-1 ura3 leu2 trp1 his3 his4*	b
RSY639	*MAT***a** *sec23-2 ura3 leu2*	c
RSY640	*MAT***a** *sec23-3 ura3 leu2*	c
RSY424	*MAT***a** *sec23-4 ura3 leu2 tyr1 lys2 ade6*	c
RDM7-4B	*MATα sec61-1 ura3 leu2 trp1 his4*	c
CSY150	*MATα sec61-3 ura3 leu2 trp1*	c
TOY221	*MATα sar1::HIS3 pep4::ADE2 ura3 leu2 trp1 his3 ade2 lys2*/pMYY3-1 (YCp[*SAR1 TRP1*])	d
TOY224	*MATα sar1::HIS3 pep4::ADE2 ura3 leu2 trp1 his3 ade2 lys2*/pMYY3-9 (YCp[*sar1*E112K *TRP1*])	d
TOY223	*MATα sar1::HIS3 pep4::ADE2 ura3 leu2 trp1 his3 ade2 lys2*/pMYY3-7 (YCp[*sar1*D32G *TRP1*])	d
TOY226	*MATα sar1::HIS3 pep4::ADE2 ura3 leu2 trp1 his3 ade2 lys2*/pMYY3-5 (YCp[*sar1*N132I *TRP1*]) and pMYU4-1 (YCp[*GAL-SAR1 URA3*])	d
YSY20	*MATα sar1::HIS3 pep4::ADE2 ura3 leu2 trp1 his3 ade2 lys2*/pMYU4-1 (YCp[*GAL1-SAR1 URA3*])	d
YSY37	*MATα eks1::TRP1 ura3 leu2 trp1 his3 ade2 lys2*	d
GMY2-12	*MATα sed4::HIS3 ura3 leu2 trp1 his3 ade2 lys2*	e
SNH023-7D	*MATα rer2-2 mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 leu2 trp1 his3 ade2 lys2*	f
SKY1	*MATα mfα1::ADE2 mfα2::LEU2 bar1::HIS3 ura3 leu2 trp1 his3 ade2 lys2*	f
SNY9	*MATα mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 leu2 trp1 his3 ade2 lys2*	f
YSY59-12A	*MATα eks1::TRP1 mfα1::ADE2 mfα2::LEU2 bar1::HIS3 ura3 leu2 trp1 his3 ade2 lys2*	d

a, Sikorski and Hieter, 1989; b, M. Bernstein (University of California, Berkeley, CA); c, R. Schekman (University of California, Berkeley, CA); d, this study; e, K. Nishimura (The University of Tokyo); f, Sato *et al.* 1995.

stain Eks1-3HAp and BiP, the samples were first treated with the 16B12 monoclonal anti-HA antibody and the polyclonal anti-BiP antibody. After washing, the decoration of the first antibodies was performed by the addition of the biotinylated goat anti-mouse IgG antibody and rhodamine-conjugated goat anti-rabbit IgG followed by streptavidin-fluorescein.

Assay of BiP Secretion—To compare the BiP secretion from the *Δeks1* cells with that of control cells under a similar genetic background, strain YSY59-12A was constructed by mating of YSY37 (*Δeks1*) and SKY1 (*26*) and tetrad dissection. BiP secreted into the culture medium was collected and analyzed by immunoblotting as described previously (*27*).

RESULTS

Identification of Multicopy Suppressors of sar1 ts Mutants—Overexpression of the *SAR1* gene suppresses three ts mutants, *sec12, sec16*, and *sec23* (*3, 6*). To exploit further genetic interactions around *SAR1*, we examined the reverse situation, that is, whether the overexpression of any related genes could suppress the ts growth of *sar1* mutants, *sar1*E112K, *sar1*D32G, and *sar1*N132I.

We first realized that pSEC1310 and pSEC1610, the original genomic clones containing *SEC13* and *SEC16*, respectively, weakly suppressed the *sar1*E112K mutant. However, the DNA fragments containing genuine *SEC13* or *SEC16* alone did not suppress this ts mutant. Considering the possibility that novel suppressor genes may be present in the original inserts, we examined the sequence of these fragments in detail. As shown in Fig. 1A, the insert of pSEC1310 contained not only *SEC13* but also a hypothetical open reading frame YLR207w. Subclones containing either of these ORFs were constructed on a multicopy plasmid and introduced into the *sar1*E112K mutant. The *Eco*RV-*Sal*I (in the vector) fragment containing the full-length *SEC13* gene no longer showed the suppressor activity. In contrast, the *Hin*dIII-*Nsp*V fragment containing the ORF YLR207w still suppressed *sar1*E112K. Thus we concluded that this ORF was the authentic suppressor gene and gave it a novel name, *EKS1* (*sar1*E112K Suppressor). *EKS1* also suppressed *sar1*D32G (Fig. 2). In the meantime, Hampton *et al.* (*28*) identified the same ORF as *HRD3*, a gene implicated in the degradation of Hmg2p.

The insert of pSEC1610, on the other hand, contained no complete ORF. Sequencing of pSEC1610 revealed a 5′-truncated version of *SEC16* (837-2194 amino acid residues) and 3′-truncated *BRO1* (1-504 amino acid residues) (*29*) (Fig. 1B). The *Kpn*I-*Nhe*I (vector) fragment containing the 3′-truncated *BRO1* did not suppress *sar1*E112K, and thus we concluded that it was the 5′-truncated *SEC16* that had the suppression activity. This 5′-truncated *SEC16* was probably expressed from a promoter in the vector and was in fact translated (see below). The truncated *SEC16* did not suppress *sar1*D32G or *sar1*N132I. The reason why the full length *SEC16* did not suppress *sar1*E112K was probably because its overexpression had a deleterious effect on cells (*14*), which concealed the suppression. The deletion of the 5′ region eliminated such toxicity.

We also found that *SEC12* could suppress *sar1*E112K but not *sar1*D32G or *sar1*N132I on a multicopy plasmid.

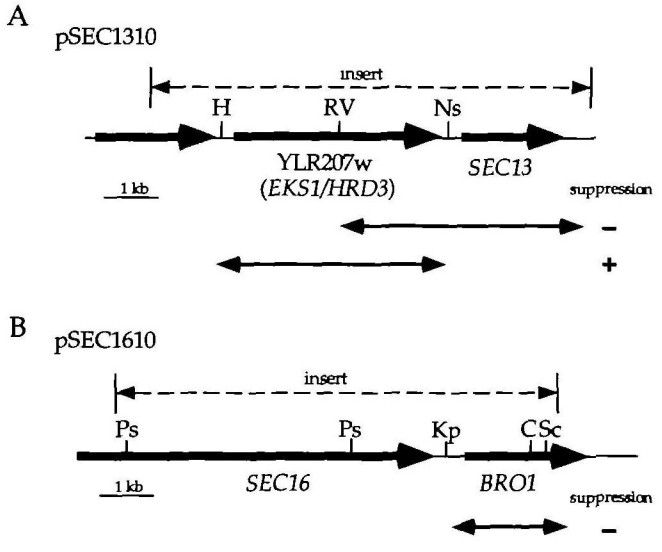

Fig. 1. **Restriction maps of the genomic clones that suppressed *sar1*E112K.** (A) pSEC1310 contained two full-length ORFs: *SEC13* and YLR207w. The suppression activities of subclones toward *sar1*E112K are also shown. We named this latter ORF *EKS1* for the *sar1*E112K suppressor. It is identical to *HRD3* reported by Hampton *et al.* (*28*). (B) pSEC1610 contained two incomplete ORFs: 5′-truncated *SEC16* and 3′-truncated *BRO1*. The restriction sites are: H, *Hin*dIII; RV, *Eco*RV; Ns, *Nsp*V; Ps, *Pst*I; Kp, *Kpn*I; C, *Cla*I; Sc, *Sac*I.

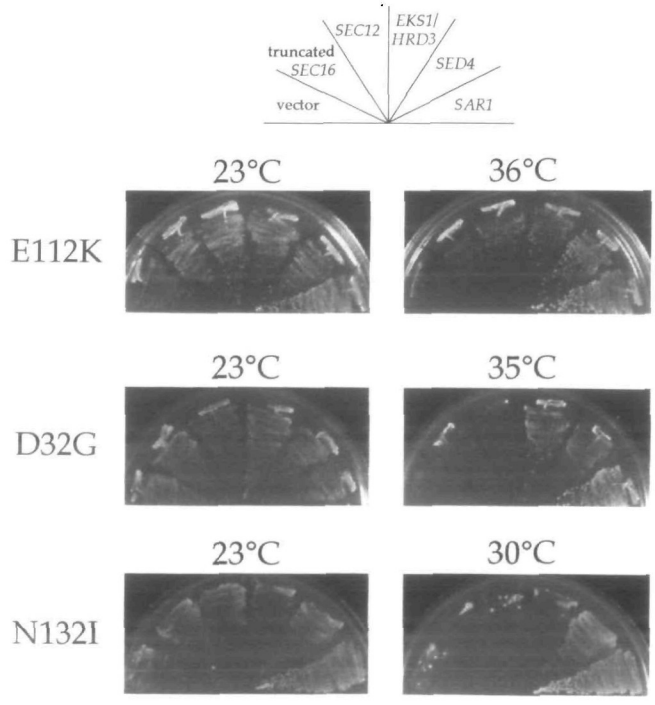

Fig. 2. **Four multicopy suppressors of *sar1* ts mutants.** *sar1* E112K (TOY224), *sar1*D32G (TOY223), and *sar1*N132I (TOY226) cells were transformed with *SAR1* (pYOSAR), *SED4* (pANY4-2), *EKS1/HRD3* (pYS112KS-1), *SEC12* (pYO12), 5′-truncated *SEC16* (pSEC1610) and vector (pYO325) on a multicopy plasmid. The transformants were incubated at the indicated temperatures for 4 days (*sar1*E112K), 5 days (*sar1*D32G), and 11 days (*sar1*N132I) on MVD plates.

Since *SAR1* is a multicopy suppressor of the *sec12* ts mutant, this is the "*vice versa*" relationship. In the yeast chromosome III, a structural homologue of *SEC12* is present (ORF YCL67c). We obtained a λ phage containing this ORF from the ordered clone bank made by Yoshikawa and Isono (*21*) and subcloned it. This gene was later identified independently as a multicopy suppressor of the deletion of *ERD2* (*30*) and the *sec16* ts mutant (*31*) and named *SED4*. We examined whether *SED4* also had a suppression activity toward *sar1* ts mutants and found that it suppressed all alleles of these mutants.

All these results are summarized in Fig. 2. Four genes, 5′-truncated *SEC16*, *SEC12*, *EKS1*, and *SED4*, suppressed the ts growth of *sar1*E112K when they were on a multicopy plasmid, and two of them, *EKS1* and *SED4*, did so for *sar1*D32G. *sar1*N132I was suppressed only by *SED4*. For *sar1*E112K, *SED4* showed the strongest suppression among the four genes. Besides these four genes, *SEC24* showed very weak suppression toward *sar1*E112K (data not shown). Other *SEC* genes we examined, *SEC7*, *SEC13*, *SEC23*, and *SEC31*, did not show any notable effects.

For *EKS1*, we examined whether this gene could suppress other *sec* mutants on a multicopy plasmid. We tested *sec12-4*, *sec13-1*, *sec16-2*, *sec23-1*, *sec23-2*, *sec23-3*, *sec23-4*, *sec20-1*, *sec21-1*, *sec22-3*, *sec61-1*, and *sec61-3* ts mutant cells as listed in Table I, but did not observe any effect. The overexpression of *EKS1* also did not suppress the lethality of *Δsar1*. That the multicopy *EKS1* not only suppresses the ts growth of *sar1*E112K but also restores secretion was confirmed by the examination of invertase secretion on a bromocresol-purple (BCP)/sucrose plate (*27*) (data not shown).

EKS1 Encodes an ER Transmembrane Protein—EKS1 encodes a protein of 833 amino acid residues with the molecular mass of 95 kDa. Computer analysis revealed two hydrophobic stretches, a potential signal peptide (SP) at the N-terminus, and a transmembrane domain (TM) in the C-terminal region (Fig. 3A). This predicts that Eks1p is a type-I transmembrane protein with the large N-terminal domain facing the ectoplasmic side (lumen of the ER) and the short C-terminal tail remaining in the cytoplasm. Eks1p has five potential *N*-glycosylation sites before the putative transmembrane domain and none in the C-terminal tail. If the N-terminal domain of Eks1p is in fact translocated into the lumen of the ER, these sites may well be glycosylated.

To test this hypothesis, Eks1p was tagged with the HA epitope at the N-terminus. A DNA fragment encoding three tandem repeats of the HA sequence was inserted right behind the putative signal peptide (Fig. 3A). This tagged version of *EKS1* (*EKS1-3HA*) is functional because its suppression of *sar1*E112K was as good as that of the wild-type gene (Fig. 3B). The lysate was prepared from the *sar1*E112K cells expressing *EKS1-3HA* and analyzed by immunoblotting using an anti-HA monoclonal antibody. As shown in Fig. 3C, Eks1-3HAp was detected as a single band migrating with the apparent molecular mass of 100 kDa. Treatment with endoglycosidase H (endo H) increased the mobility of this band by about 3 kDa, indicating that one or two of the potential *N*-glycosylation sites were indeed glycosylated. Thus, we conclude that Eks1p is a type-I transmembrane glycoprotein with a short cytoplasmic tail at the C-terminus. Another interesting feature of Eks1p is a cluster of 9 glutamine residues in the C-terminal tail (Fig. 3A, Q-rich). Its functional significance is unknown at present.

The intracellular localization of Eks1p was examined by immunofluorescence microscopy. The *eks1* disruptant cells (see below) carrying *EKS1-3HA* on a single-copy plasmid

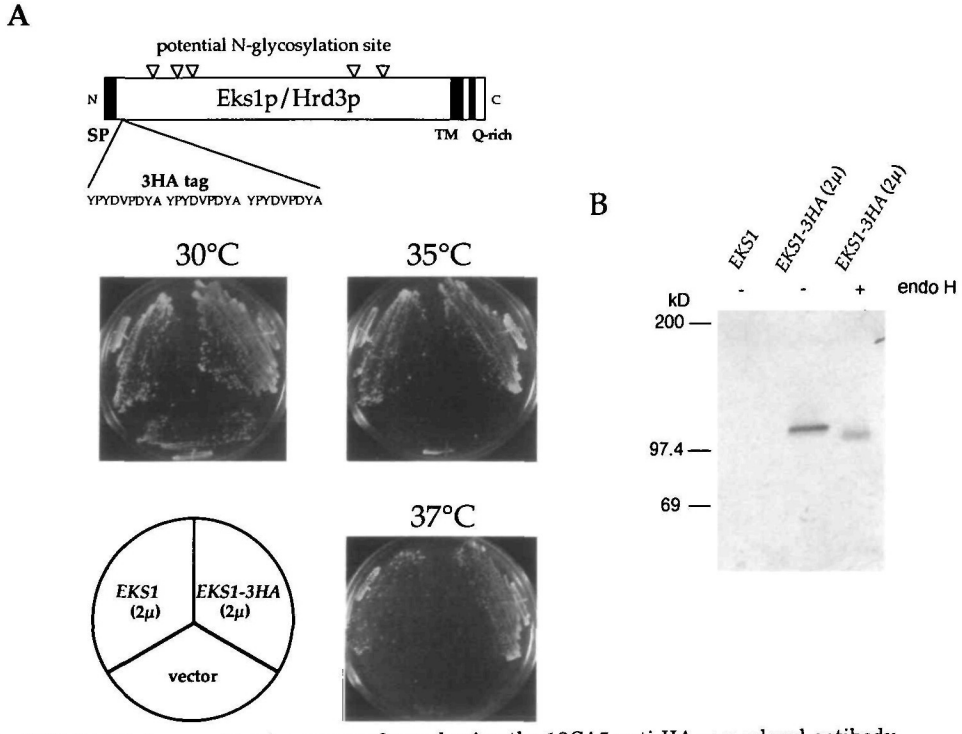

Fig. 3. ***EKS1* encodes a type-I transmembrane protein.** (A) Schematic diagram of the *EKS1* gene product (Eks1p). Eks1p has two hydrophobic stretches, a putative signal peptide (SP) at the N-terminus, and a transmembrane domain (TM) near the C-terminus. A cluster of glutamine residues (Q-rich) exists in the C-terminal tail. Potential *N*-glycosylation sites are indicated by arrowheads. The site of insertion of the 3HA epitope tag is also shown. (B) 3HA-tagged *EKS1* suppressed the *sar1*E112K mutant as well as the wild-type *EKS1* did. Multicopy plasmids harboring *EKS1-3HA* (pYS15), wild-type *EKS1* (pYS3), and vector alone (pSQ326) were introduced into the *sar1*E112K cells (TOY-224). The transformants were incubated at indicated temperatures for 3 days. (C) Eks1p is glycosylated. The *sar1*E112K cells (TOY224) carrying *EKS1* (pYS3) or *EKS1-3HA* (pYS15) were grown at 30°C and harvested at the early log phase. Cell lysates were prepared, treated, or not treated with endoglycosidase H (endo H), and resolved by SDS-PAGE. Immunoblotting was performed using the 12CA5 anti-HA monoclonal antibody.

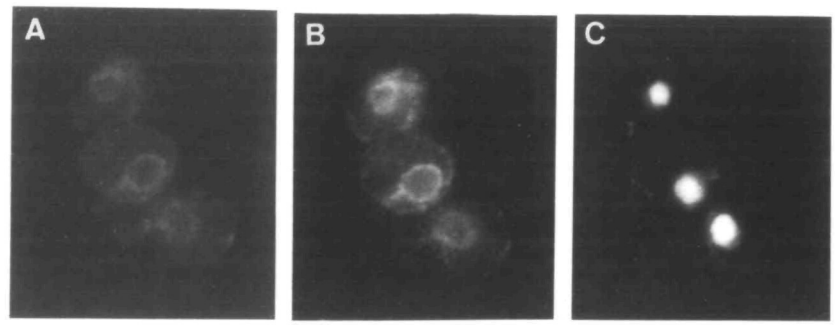

Fig. 4. **Eks1p is located in the ER.** The *eks1*-disrupted cells (YSY37) carrying *EKS1-3HA* on a single-copy plasmid (pYS14) were prepared for double indirect immunofluorescence with the anti-BiP polyclonal antibody (A) and the 16B12 anti-HA monoclonal antibody (B). Panel C shows the staining with 4′,6-diamidiano-2-phenylindole to localize nuclei.

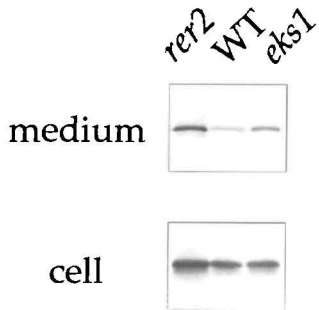

Fig. 5. **The *eks1*-deleted cells missecrete an ER resident protein, BiP, to the medium.** Cells of the *rer2-2* mutant (SNH023-7D), wild type (SNY9), and the *eks1* disruptant (YSY59-12A) were grown at 23°C in YPD medium. The culture media were collected and proteins were precipitated by the addition of 4 volumes of acetone. The remaining cell pellet was disrupted with glass beads to prepare cell lysates. Both media and lysates were analyzed by SDS-PAGE and immunoblotting with the anti-BiP antibody.

were double stained with the 16B12 anti-HA monoclonal antibody and the anti-BiP antibody. As shown in Fig. 4, the staining by the two antibodies exhibited very good colocalization and labeled the ER structures, nuclear envelopes, and peripheral ER. Thus, Eks1p resides in the ER.

The Disruption of EKS1—The *EKS1* gene was knocked out in diploid yeast cells, which was then subjected to tetrad dissection. All four spores grew indicating that the *eks1* disrupted cells (*Δeks1*) are viable. We examined the growth of *Δeks1* cells in detail from 15 to 37°C but did not find any defects as compared to the wild type (data not shown). The intracellular protein transport was also examined by looking at carboxypeptidase Y, invertase, Gas1p, and α-factor precursor as marker proteins. Careful pulse-chase experiments at various temperatures, however, did not reveal any lesions in their transport (not shown). The only phenotype we could find with *Δeks1* cells was the missecretion of BiP. An ER-resident protein, BiP (Kar2p), is normally localized to the ER, but is known to be secreted into the medium in several mutants (*erd1, erd2, rer1, rer2,* and *emp24/bst2*) (*26, 27, 32-34*). As shown in Fig. 5, the *Δeks1* cells secreted an elevated amount of BiP, although it was not as remarkable as *rer2* cells. There was no significant difference in the cellular level of BiP between the wild-type and *Δeks1* cells.

The Role of Eks1p/Hrd3p on the Stability of Sar1p and Sec Proteins—While our study on *EKS1* was in progress, Hampton *et al.* (*28*) reported that mutations in three genes increased the stability of Hmg2p, an isozyme of HMG-CoA

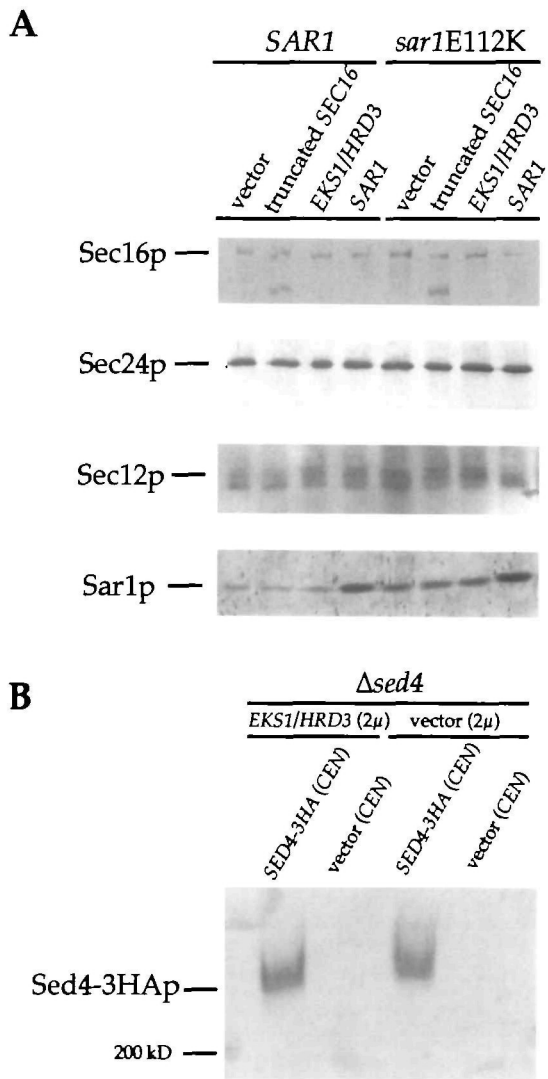

Fig. 6. **The overexpression of *EKS1/HRD3* does not affect the levels of Sar1p or the products of multicopy suppressors of *sar1*.** (A) Wild-type (TOY221) and *sar1*E112K (TOY224) cells harboring 5′-truncated *SEC16* (pSEC1610), *EKS1/HRD3* (pYS112-KS-1), *SAR1* (pYOSAR) on a multicopy plasmid, or vector alone (pYO325) were subjected to the immunoblotting analysis using antibodies against Sec16p, Sec24p, Sec12p, and Sar1p. (B) The *sed4* disruptant cells (GMY2-12) harboring *SED4-3HA* (pYS28-E52) or vector (pRS314) on a single-copy plasmid were further transformed with *EKS1/HRD3* on a multicopy plasmid (pYS3) or vector alone (pSQ326) and subjected to immunoblotting using the 16B12 anti-HA monoclonal antibody.

reductase. One of them, *HRD3*, was identical to *EKS1*. In the *hrd3* mutant cells, the degradation of Hmg2p was slowed down and the steady-state level of Hmg2p increased significantly. Interestingly, the overproduction of *HRD3* appears also to have the effect of stabilizing Hmg2p (R. Hampton, personal communication).

If Eks1p/Hrd3p is in fact involved in ER protein degradation and its imbalance with other components causes the stabilization of ER proteins, our observation of *sar1* suppression by multicopy *EKS1/HRD3* might be explained by the stabilization of Sar1p itself. To test this possibility, we investigated the intracellular levels of Sar1p by immunoblotting. As shown in Fig. 6A, the steady-state levels of wild-type and mutant Sar1p were not affected by the overexpression of *EKS1/HRD3*. We also performed a pulse-chase experiment to follow the stability of Sar1p. For either the wild-type Sar1p or the mutants Sar1pE112K and Sar1pD32G, the life times of the proteins were all quite long (more than 10 h), and no apparent effect of Eks1p/Hrd3p overproduction or deletion was observed on them (data not shown). Another possibility is that *EKS1/HRD3* stabilizes one of the products of the multicopy suppressors of *sar1* and thus suppresses the *sar1* mutation. However, the levels of Sec16p, Sec24p, and Sec12p were not changed significantly by the overexpression of *EKS1/HRD3* or 5′-truncated *SEC16* (Fig. 6A). The amount of Sed4p was examined by expressing *SED4-3HA* in *Δsed4* cells. 3HA-tagged *SED4* was functional because its overexpression suppressed *sar1* and *sec16* as well as that of the authentic *SED4* did (data not shown). Again, the level of Sed4-3HAp was not altered by the overexpression of *EKS1/HRD3* (Fig. 6B). These results indicate that the effect of *EKS1/HRD3* overproduction on *sar1* suppression was not due to the stabilization of Sar1p or its suppressors, and argue against the model in which Eks1p/Hrd3p is involved in ER protein degradation in general.

It should be noted here that, in the lanes of Sec16p immunoblotting (Fig. 6A), a smaller band was clearly observed when the 5′-truncated *SEC16* was introduced. This indicates that this truncated gene was in fact expressed from pSEC1610.

DISCUSSION

Identification of Multicopy Suppressors of sar1 ts Alleles—Sar1p is one of the best characterized of the small GTPases that are involved in regulation of vesicular trafficking. It is essential for the formation of COPII vesicles from the ER by virtue of its role in the assembly of COPII components onto the ER membrane. Through *in vivo* and *in vitro* experiments, we have presented a model in which the GTP-bound form of Sar1p functions in the vesicle budding, and the GTP hydrolysis takes place when the vesicle formation is completed (*6, 19*). However, many questions remain to be answered. For example, what triggers the activation of Sar1p into the GTP-bound state through the Sec12p GEF, what recognizes the completion of vesicle formation and activates the GTP hydrolysis through the Sec23p GAP, and how is Sar1p-GDP recycled to the ER? To address these questions, we have taken a genetic approach involving isolation of conditional ts mutants of *SAR1* and screening of their suppressors. This article is the first report of the identification of *sar1* suppressors.

Three *sar1* ts mutants, *sar1*E112K, *sar1*D32G, and *sar1*N132I, have been isolated (*17, 18*). Two of these, *sar1*E112K and *sar1*D32G, have been categorized as GDP-form mutants (*19*). As multicopy suppressors of these alleles, four genes have been identified in this study: *SEC12*, *SED4*, truncated *SEC16*, and *EKS1/HRD3*. Their abilities to suppress *sar1* ts mutants vary with alleles: *sar1*E112K is suppressed by all four, *sar1*D32G is suppressed by *SED4* and *EKS1/HRD3*, and *sar1*N132I is suppressed only by *SED4*.

Genetic Interactions between SAR1, SEC12, SEC16, and SED4—Sec12p is the GEF of Sar1p. It is conceivable that its overexpression suppresses *sar1*E112K, which is a GDP-form mutant, by enhancing the guanine-nucleotide exchange activity. However, it did not show appreciable effects on the other GDP-form mutant, *sar1*D32G. Conversely, the overexpression of *SAR1* suppresses the *sec12-4* ts mutant very strongly (*3, 10*).

Interestingly, *SED4*, a homologue of *SEC12*, is the strongest *sar1* suppressor among the four genes we identified. Sed4p exhibits 45% identity with Sec12p in the amino acid sequences of the cytoplasmic domains (*30*) but is said to show no detectable GEF activity towards Sar1p (C. Barlowe, personal communication). It is also intriguing that a 5′-truncated form of *SEC16* suppresses *sar1*E112K, although weakly. The full-length *SEC16* was unable to suppress *sar1*E112K, but this is probably because the overexpression of the whole *SEC16* is toxic to cells (*14*). These data depict very intimate genetic relationships between the three genes, *SAR1*, *SED4*, and *SEC16*, as shown in Fig. 7. Sec16p has been shown to bind Sed4p, Sec23p, Sec24p, and Sec31p (*14, 16, 31*). The biochemical roles of Sed4p and Sec16p in the regulation of Sar1p function remain to be elucidated.

The Role of EKS1/HRD3 in the ER Function—The *EKS1/HRD3* gene also shows tight genetic interaction with *SAR1*. Its overexpression suppresses not only *sar1*E112K and *sar1*D32G but also many new ts alleles of *sar1* that we have recently isolated (our unpublished results). We have shown that *EKS1/HRD3* encodes a type-I transmembrane glycoprotein in the ER. Hampton *et al.* (*28*) reported that a mutation of *EKS1/HRD3* causes stabilization of Hmg2p, an isoform of HMG-CoA reductase, and suggested the role of Eks1p/Hrd3p in degradation of Hmg2p. Either the disruption or overexpression of *EKS1/HRD3* appears to stabilize Hmg2p, implying that the balance of the Eks1p/Hrd3p amount with that of other components is important for efficient degradation of Hmg2p. However, the data obtained in this study do not necessarily support this

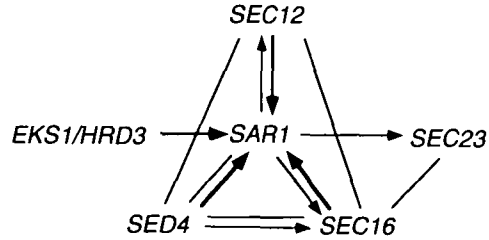

Fig. 7. **Genetic interactions around the *SAR1* gene.** Thick lines indicate the results of this study. Simple lines (no arrowheads), synthetic lethality; lines with a single arrowhead, multicopy suppression.

model. First, the overexpression of *EKS1/HRD3* does not affect the stability of wild-type Sar1p, mutant Sar1p, Sec12p, Sec16p, Sec24p, or Sed4p. The suppression effect by *EKS1/HRD3* is thus not exerted through the stabilization of Sar1p itself or its known suppressors. Second, the deletion of *EKS1/HRD3* does not change the growth phenotype of the *sec61* ts mutants (our unpublished results). The ts mutants of Sec61p are known to undergo dislocation from the ER to the cytosol and then degradation by proteasome at the restrictive temperature. Ubc6p, a ubiquitin-conjugating enzyme, plays an important role in this process, and its imbalance by either gene disruption or overexpression perturbs the efficient degradation of Sec61p and thus suppresses the temperature sensitivity of *sec61* (*35, 36*). Such suppression was not observed with *EKS1/HRD3*. Even though Eks1p/Hrd3p may still have a role in degradation or stabilization, it does not seem to be general to all ER proteins.

Because HMG-CoA reductase catalyzes the rate-limiting step of the synthesis of isoprenoids, including sterols, ubiquinone, and dolichol, it is conceivable that the suppression of *sar1* ts by the *EKS1/HRD3* overexpression is an indirect effect exerted through the alteration of the lipid composition in the ER. However, even under conditions that the overexpression of *HMG2* causes accumulation of crystalloid ER membranes, no apparent increase of sterols was observed (*37*).

At present, the phenomena revealed for the disruption or overexpression of *EKS1/HRD3* by the two groups appear to be distant. At present, we speculate that Eks1p/Hrd3p is somehow involved in the compartmentalization of ER subdomains. For example, if the overproduction of Eks1p/Hrd3p increases the number of the vesicle budding sites in the ER, this would lead to the suppression of *sar1* ts mutants. Inefficient segregation of Hmg2p may also cause retardation of its degradation. In fact, the subcellular localization of Hmg2p is quite restricted to particular subregions of the ER (*38*). The missecretion of BiP from the *eks1/hrd3*-disrupted cells may be also consistent with the idea that this gene is important for the maintenance of the ER membrane subdomains.

In summary, we have revealed new genetic interactions between *SAR1*, *SEC12*, *SEC16*, and *SED4* by the use of the *sar1* ts mutants. The role of an ER membrane protein, Eks1p/Hrd3p, in Sar1p regulation remains to be established. We are also in the process of isolation of additional multicopy suppressors of the *sar1* mutants. Many candidate clones have been obtained and their characterization is now in progress. Detailed analysis of these genes will facilitate further understanding of the components that regulate the GTPase cycle of Sar1p, and will be reported elsewhere.

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