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SSI1 Encodes a Novel Hsp70 of the *Saccharomyces cerevisiae* Endoplasmic Reticulum

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The endoplasmic reticulum (ER) of the budding yeast *Saccharomyces cerevisiae* contains a well-characterized, essential member of the Hsp70 family of molecular chaperones, Kar2p. Kar2p has been shown to be involved in the translocation of proteins into the ER as well as the proper folding of proteins in that compartment. We report the characterization of a novel Hsp70 of the ER, Ssi1p. Ssi1p, which shares 24% of the amino acids of Kar2p, is not essential for growth under normal conditions. However, deletion of *SSI1* results in cold sensitivity as well as enhanced resistance to manganese. The localization of Ssi1p to the ER, suggested by the presence of a conserved *S. cerevisiae* ER retention signal at its C terminus, was confirmed by subcellular fractionation, protease protection assays, and immunofluorescence. The *SSI1* promoter contains an element with similarity to the unfolded protein response element of *KAR2*. Like *KAR2*, *SSI1* is induced both in the presence of tunicamycin and in a *kar2-159* mutant strain, conditions which lead to an accumulation of unfolded proteins in the ER. Unlike *KAR2*, however, *SSI1* is not induced by heat shock. Deletion of *SSI1* shows a complex pattern of genetic interactions with various conditional alleles of *KAR2*, ranging from synthetic lethality to synthetic rescue. Interestingly, *SSI1* deletion strains show a partial block in translocation of multiple proteins into the ER, suggesting that Ssi1p plays a direct role in the translocation process.

Among the most ubiquitous and highly conserved proteins are the molecular chaperones, which include a 70-kDa class known as the Hsp70s. In eucaryotes, Hsp70 family members are localized to subcellular locations, including the cytosol, mitochondria, and the endoplasmic reticulum (ER), where they are involved in such diverse cellular processes as translation, translocation into organelles, and regulated proteolysis (18, 19). Hsp70 proteins are thought to exert their many cellular effects by reversibly binding to proteins which are in a nonnative state. Cycles of protein binding and release are coupled to cycles of ATP binding, hydrolysis, and nucleotide exchange. Hsp70s differentiate between native and nonnative proteins by binding preferentially to stretches of largely hydrophobic residues (5, 23), which are exposed in unfolded proteins or unassembled multimeric complexes but are normally buried in native protein conformations. This binding is thought to prevent or reverse the aggregation or misfolding of nonnative proteins.

Hsp70 proteins are aided in these functions by their interactions with cofactors. In *Escherichia coli*, the Hsp70 protein DnaK interacts with two cofactors, DnaJ and GrpE. DnaJ can bind to substrate proteins in the absence of DnaK and may serve in some instances to target DnaK to its substrate molecules (30, 35, 60). DnaJ can also function to stimulate ATP hydrolysis by DnaK, while GrpE functions as a nucleotide exchange factor (37). Although eucaryotic GrpE homologs have so far been identified only in mitochondria, DnaJ homologs which localize to various cellular locations have been found, including two ER-localized proteins in *Saccharomyces cerevisiae*, Scj1p (58) and Sec63p (54, 55).

The ER Hsp70 of the budding yeast *S. cerevisiae* is known alternatively as yeast BiP (45), for its homology to the immunoglobulin-binding protein of mammalian cells, or Kar2p, for its role in nuclear fusion during mating or karyogamy (51). *KAR2* is an essential gene in *S. cerevisiae* whose high-level constitutive expression can be further induced by either heat shock or treatment with tunicamycin, which prevents glycosylation in the ER and thus leads to an accumulation of aberrantly folded proteins in that compartment.

In mammalian cells, BiP associates strongly with misfolded, underglycosylated, or unassembled proteins in the ER (for reviews, see references 6 and 24). *S. cerevisiae* strains carrying conditional alleles of *KAR2* have been shown to be defective in the folding of the vacuolar protein carboxypeptidase Y (CPY) in the ER. In these strains, more CPY was found in association with Kar2p than in wild-type strains, which fold CPY normally (64). Together, these data suggest that Kar2p and its mammalian homolog play a role in the folding of proteins in the ER and in the prevention of aggregation of misfolded proteins.

In addition to assisting protein folding, Kar2p plays a critical role in the ER by facilitating protein translocation across the ER membrane (for a review, see reference 11). The presence of both Kar2p in the lumen of the ER and the DnaJ homolog Sec63p in the ER membrane is required for translocation. The region of highest homology between Sec63p and DnaJ is the J domain, which is responsible for interaction between DnaJ and DnaK. The J domain of Sec63p is exposed to the ER lumen and is thought to interact with Kar2p, and a mutation which causes a single-amino-acid substitution in the J domain of Sec63p, *sec63-1*, results in a translocation defect similar to that caused by a *kar2* conditional allele (38). This defect can be overcome by the isolation of dominant suppressors in *KAR2* (38) or by replacement of the altered J domain with the corresponding wild-type region from the other known ER-localized DnaJ homolog, Scj1p (58).

The mechanism of action of Kar2p and Sec63p in translocation remains to be clarified. To enter the ER, proteins transit a pore consisting at least in part of the essential integral membrane protein Sec61p. The pore is too narrow to accommodate native protein structures, and proteins are instead translocated in an extended conformation, often cotranslationally. By anal-

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TABLE 1. Yeast strains used in this study

Description	Strain	Genotype	Source and/or reference
sec62	RSY529	*MAT*α *leu2-3,112 his4 ura3-52 sec62*	R. Schekman
Δ*ssi1*/$^+$	BB257	*MAT***a**/α *trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100 GAL2$^+$/GAL2$^+$ met2-Δ1/met2-Δ1 lys2-Δ2/lys2-Δ2 Δssi1::LEU2/$^+$*	This study
Δ*ssi1*	BB258	*MAT*α *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2$^+$ met2-Δ1 lys2-Δ2 Δssi1::LEU2*	This study
SSI1	BB259	*MAT***a** *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2$^+$ met2-Δ1 lys2-Δ2*	This study
SSI1	BB260	*MAT*α *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2$^+$ met2-Δ1 lys2-Δ2*	This study
Δ*ssi1*	BB261	*MAT***a** *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2$^+$ met2-Δ1 lys2-Δ2 Δssi1::LEU2*	This study
kar2-1	MS1000	*MAT*α *ura3-52 trp1-Δ1 kar2-1*	M. Rose (61)
kar2-113	MS544	*MAT*α *ura3-52 trp1-Δ1 kar2-113*	M. Rose (61)
kar2-133	MS553	*MAT*α *ura3-52 trp1-Δ1 kar2-133*	M. Rose (61)
kar2-159	MS137	*MAT***a** *ura3-52 leu2-3,112 ade2-101 kar2-159*	M. Rose (71)
kar2-191	MS1327	*MAT*α *ura3-52 trp1-Δ1 kar2-191*	M. Rose (61)
kar2-1/$^+$ Δ*ssi1*/$^+$	BB327	*MAT***a**/α *trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ade2-1/ade2-1 met2-Δ1/met2-Δ1 lys2-Δ2/lys2-Δ2 Δssi1::LEU2/$^+$ kar2-1/$^+$*	This study
kar2-113/$^+$ Δ*ssi1*/$^+$	BB306	*MAT***a**/α *trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ade2-1/ade2-1 met2-Δ1/met2-Δ1 lys2-Δ2/lys2-Δ2 Δssi1::LEU2/$^+$ kar2-113/$^+$*	This study
kar2-133/$^+$ Δ*ssi1*/$^+$	BB319	*MAT***a**/α *trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ade2-1/ade2-1 met2-Δ1/met2-Δ1 lys2-Δ2/$^+$ Δssi1::LEU2/$^+$ kar2-133/$^+$*	This study
kar2-159/$^+$ Δ*ssi1*/$^+$	BB307	*MAT***a**/α *trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ade2-1/ade2 met2-Δ1/met2-Δ1 lys2-Δ2/lys2-Δ2 Δssi1::LEU2/$^+$ kar2-159/$^+$*	This study
kar2-191/$^+$ Δ*ssi1*/$^+$	BB333	*MAT***a**/α *trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/$^+$ ade2-1/ade2 met2-Δ1/met2-Δ1 lys2-Δ2/lys2-Δ2 Δssi1::LEU2/$^+$ kar2-191/$^+$*	This study
SSI1	BB338	*MAT***a** *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 met2-Δ1 lys2-Δ2*	This study
Δ*ssi1*	BB339	*MAT*α *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2 met2-Δ1 lys2-Δ2 Δssi1::LEU2*	This study
kar2-1	BB340	*MAT*α *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 met2-Δ1 lys2-Δ2 kar2-1*	This study
kar2-1 Δ*ssi1*	BB341	*MAT*α *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 met2-Δ1 lys2-Δ2 kar2-1 Δssi1::LEU2*	This study
kar2-133	BB342	*MAT***a** *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 met2-Δ1 kar2-133*	This study
kar2-133 Δ*ssi1*	BB343	*MAT*α *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 met2-Δ1 lys2-Δ2 kar2-133 Δssi1::LEU2*	This study
kar2-191	BB344	*MAT*α *trp1-1 ura3-1 leu2-3,112 ade2 met2-Δ1 lys2-Δ2 kar2-191*	This study
kar2-191 Δ*ssi1*	BB345	*MAT*α *trp1-1 ura3-1 leu2-3,112 ade2 met2-Δ1 lys2-Δ2 kar2-191 Δssi1::LEU2*	This study
Δ*ssi1*/$^+$	BB351	*MAT***a**/α *Δtrp1/Δtrp1 ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 GAL2$^+$/GAL2$^+$ lys1/lys1 lys2/lys2 Δssi1::LEU2/$^+$*	This study
Δ*ssi1*	BB352	*MAT*α *Δtrp1 ura3-52 leu2-3,112 his3-11,15 GAL2$^+$ lys1 lys2 Δssi1::LEU2*	This study
SSI1	BB353	*MAT***a** *Δtrp1 ura3-52 leu2-3,112 his3-11,15 GAL2$^+$ lys1 lys2*	This study
SSI1	BB354	*MAT*α *Δtrp1 ura3-52 leu2-3,112 his3-11,15 GAL2$^+$ lys1 lys2*	This study
Δ*ssi1*	BB355	*MAT***a** *Δtrp1 ura3-52 leu2-3,112 his3-11,15 GAL2$^+$ lys1 lys2 Δssi1::LEU2*	This study

ogy to the action of the essential yeast mitochondrial Hsp70, Ssc1p, in facilitating translocation into that compartment (26, 66), Kar2p may simply bind to the translocating precursor and prevent its back-slipping through the translocation pore or Kar2p may exert a more active role by using nucleotide-dependent conformational changes as a motor to pull precursors into the ER. Sec63p may catalyze sequential cycles of ATP hydrolysis by Kar2p or may serve to target Kar2p to the translocation apparatus.

Here, we report the characterization of a novel Hsp70 of the endoplasmic reticulum, Ssi1p, which we suggest plays a role in the translocation of proteins into the ER. *SSI1* expression and *KAR2* expression are similarly regulated, and *SSI1* is essential for viability in certain *kar2* mutant backgrounds. Although *SSI1* is not required for growth under normal conditions, the translocation of proteins into the ER is partially blocked in *SSI1* deletion strains. These findings have implications for further understanding the role of Kar2p and other factors in the translocation process.

MATERIALS AND METHODS

Strains and media. The yeast strains used in this study are described in Table 1. Strains carrying the *sec12*, *sec13*, *sec16*, *sec18*, *sec20*, *sec21*, *sec23*, *sec61*, and *sec63* alleles, used in preliminary genetic analyses, were obtained by crossing the parent *sec* strains (a gift from R. Schekman) into another background to obtain isolates which carry the *sec* alleles in the presence of auxotrophic markers (42). Strains BB257 and BB351 (Table 1) were obtained by transformation of wild-type diploids with a Δ*ssi1* disruption construct (see below). BB257 is a derivative of wild-type strain PJ53, which was derived from W303 (69) with the addition of auxotrophic markers (31a). BB351 is derived from BB350, a diploid derivative of wild-type strain DS110 (31, 65). DS110 is a derivative of S288C. BB258, BB259, BB260, and BB261 are the progeny of BB257 and were obtained from four spores of a single ascus. Similarly, BB352, BB353, BB354, and BB355 were obtained from four spores of a single ascus of BB351.

Diploids which were heterozygous for a conditional *kar2* allele and for deletion of *SSI1* were obtained by crossing a *kar2* mutant strain (MS1000, MS544, MS553, MS137, or MS1327 [Table 1]) with a Δ*ssi1* strain (either BB258 or BB261) and then backcrossing a *kar2* isolate from this diploid to one of the Δ*ssi1* haploids three times to minimize the effects of different strain backgrounds. Diploids from the third backcross in each case are shown in Table 1.

As described below, heterozygous diploids were transformed with p316-SSI1, sporulated, and dissected. Ura$^-$ progeny of the indicated genotypes were selected on 5′-fluoroorotic acid (5′-FOA). Diploid parents were as follows: BB338 (wild type) arose from BB307; BB339 (Δ*ssi1*) arose from BB306; BB340 and

BB341 arose from BB327 (*kar2-1*); BB342 and BB343 arose from BB319 (*kar2-133*); and BB344 and BB345 arose from BB333 (*kar2-191*).

Unless otherwise indicated, yeast strains were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) or minimal medium (0.67% yeast nitrogen base without amino acids, 2% dextrose, supplemented with required amino acids as necessary) at 30°C. A modified lithium acetate protocol was used for yeast transformations (25). *E. coli* cells were grown in LB (0.5% yeast extract, 1% tryptone, 1% NaCl) supplemented with 100 μg of ampicillin per ml as necessary for plasmid selection and were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's instructions or by a $CaCl_2$-based protocol (39). Restriction enzymes and buffers were from New England Biolabs (Beverly, Mass.), Promega (Madison, Wis.), or Boehringer Mannheim (Indianapolis, Ind.).

Identification of *SSI1*. A yeast sequence database search for Hsp70 homolog genes was performed by using the yeast protein Ssa1p sequence as a query sequence in the BLASTp search algorithm. This search identified an open reading frame on chromosome 11 (GenBank accession no. Z28073; sequenced as part of the yeast sequencing project [49]) predicted to encode an 881-amino-acid protein with homology to known hsp70 proteins. We have named this gene *SSI1*.

Disruption of *SSI1*. In order to disrupt the *SSI1* gene, a 1,571-bp PCR fragment containing the N-terminal 1,173 bp of *SSI1* was amplified from genomic DNA with primers P1 (5′AAAAGGTACCGGAAGCTCACCGTCAC3′) and P2 (5′-AAAAGAGCTCCCGCCAAAATGACCC3′). The *SSI1* PCR product and plasmid pRS304 (63) were each cut with *Kpn*I and *Sac*I (whose sites are underlined in the primer sequences) and ligated together to obtain plasmid pRS304-SSI1PCR. *LEU2* was excised from YEp13 as a 2.8-kb *Bgl*II fragment and ligated into the *Bam*HI site of pRS304 to produce pRS304-LEU2. A 774-bp *Pst*I-*Stu*I fragment from pRS304-SSI1PCR containing the N-terminal 732 bp of the open reading frame of *SSI1* was excised and replaced with the *LEU2* gene on a *Pst*I-*Ecl*136 fragment from pRS304-LEU2 to produce pΔSSI1::LEU2. A 1,297-bp *Bss*HII-*Sac*I linear fragment from pΔSSI1::LEU2 which contained the *LEU2* marker flanked by 83 bp of the 5′ noncoding sequence of *SSI1* and 251 bp from the middle of the *SSI1* open reading frame was transformed into a diploid yeast strain, and the disruption was confirmed by Southern blot analysis.

Cloning and subcloning of *SSI1*. *E. coli* RR1 cells were transformed with DNA from a YCp50-based genomic DNA library (52), and approximately 4,000 colonies (at a density of approximately 800 per plate) were transferred onto nylon filters. Probes for colony filter hybridization were prepared by random priming essentially as previously described (2) using the 1,571-bp *SSI1* double-stranded PCR product as a template and were hybridized to filters under stringent conditions. Colonies corresponding to a positive signal were isolated, and single colonies were purified by two additional rounds of colony hybridization. Plasmid DNA was prepared, and the presence of the genomic clone in isolates was confirmed by restriction digest mapping and Southern blot analysis.

SSI1 was subcloned from the isolated genomic clone in three steps. First, a 2.5-kb *Hin*dIII fragment containing the 3′-terminal 143 bp of *SSI1* was cloned into the multicopy, *URA3*-marked vector pRS426 (16). Then a 1.3-kb *Eco*RI fragment from the resulting plasmid was excised, and the plasmid was religated to remove the downstream *Hin*dIII site, leaving the 3′-terminal 143 bp of *SSI1* and 1,055 bp of the downstream flanking region. Finally, a 3.6-kb *Hin*dIII fragment from the genomic clone containing the N-terminal 2,499 bp of *SSI1* and a 1,094-bp 5′ flanking region was ligated into the remaining *Hin*dIII site to produce p426-SSI1. To create a centromeric version of this construct, the insert was removed from p426-SSI1 as a 4.8-kb *Sal*I-*Bam*HI fragment and ligated into the centromeric, *URA3*-marked vector pRS316 (63).

Generation of antibodies against Ssi1p. Plasmid pGEXΔB-SSI1-RH, which encodes a fusion of glutathione *S*-transferase to amino acids 513 to 835 of Ssi1p, was constructed as follows. pGEX-KG (27) was digested with *Bam*HI. The overhanging ends were filled in with the Klenow fragment of DNA polymerase I, and the plasmid was religated to give pGEXΔB. A 1-kb *Eco*RI-*Hin*dIII fragment of p426-SSI1 was ligated into pGEXΔB to give pGEXΔB-SSI1-RH.

A 2-liter culture of *E. coli* MC1066Δ was grown to mid-log phase in T broth (1.6% tryptone, 0.5% NaCl) at 30°C and induced for 100 min with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested, resuspended in phosphate-buffered saline (PBS)–Triton (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% Triton X-100), and lysed by French press extraction. A cleared lysate (32,000 × *g* supernatant) was incubated with preswollen glutathione-agarose beads (Sigma Chemical Co., St. Louis, Mo.). The fusion protein was eluted with 10 mM glutathione, and this eluate was used to inoculate rabbits.

Immunoblot analysis. For immunoblot analysis, yeast cells were lysed by vortexing with glass beads in breaking buffer (2% Triton X-100, 1% sodium dodecyl sulfate [SDS], 100 mM NaCl, 10 mM Tris [pH 8.0], 1 mM EDTA) and boiled in SDS sample buffer (7). Lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE), stained with Coomassie brilliant blue, and quantitated by densitometric scanning to normalize loadings. Then equivalent loadings were separated by SDS-PAGE and transferred to nitrocellulose (Hybond-C; Amersham Corp., Arlington Heights, Ill.). For Ssi1p immunoblots, membranes were blocked for at least 1 h in TBS-Tween (150 mM NaCl, 20 mM Tris [pH 7.4], 0.5% Tween 20) with 3% nonfat dry milk. Polyclonal antibodies against Ssi1p were diluted 1:500 in the same buffer and incubated with membranes for at least 1 h. Goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Amersham) were diluted 1:10,000 and incubated with membranes for at least 30 min. The results were visualized by enhanced chemiluminescence (Amersham). For Kar2p, alpha factor, and Hsp104 immunoblots, membranes were blocked without milk and the secondary antibodies were diluted 1:40,000. Kar2p antibodies (a gift from M. Rose) were diluted 1:3,000; Hsp104 antibodies (a gift from S. Lindquist) were diluted 1:10,000.

Subcellular fractionation. Wild-type cells (strain BB259) were grown to mid-log phase in 1 liter of YPD. Cells were harvested, washed once with water, resuspended in 100 mM Tris-SO_4 (pH 9.4) with 10 mM dithiothreitol (DTT), and incubated for 10 min at 30°C. DTT-treated cells were harvested, washed once with 1.2 M sorbitol, resuspended in 1.2 M sorbitol–20 mM KH_2PO_4 (pH 7.4), and spheroplasted with 3 mg of Zymolyase 20T (ICN Biomedicals, Irvine, Calif.) per g of cells for 30 min at 30°C. Spheroplasts were washed once with 1.2 M sorbitol and resuspended in lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 7.5], 500 mM sucrose, 3 mM magnesium acetate, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride). Subcellular fractions were prepared essentially as previously described (14). Briefly, lysates were prepared with a Dounce homogenizer and cleared by spinning at 10,000 × *g*. A portion of the cleared lysate was spun at 100,000 × *g* for 1 h at 4°C in a TL-100 ultracentrifuge (Beckman Instruments, Fullerton, Calif.). The supernatant from this spin corresponds to the soluble fraction. To obtain the microsomal fraction, an aliquot of cleared lysate was layered on top of a 35% Percoll solution which was then spun in the ultracentrifuge at 76,000 × *g* for 50 min at 4°C. The upper of two turbid bands was collected and corresponds to the microsomal fraction. Immunoblot analysis of the fractions was performed as described above. Loadings were adjusted to give equal quantities of Kar2p, a known ER resident protein, in the cleared-lysate and microsomal fractions.

Protease protection assays. Uncleared homogenates were prepared as described in the fractionation protocol above, with the omission of protease inhibitors. Aliquots of homogenate were treated with either water or 0.5% Triton X-100 and then digested with 10 to 25 μg of proteinase K per ml or mock digested with water. At the time points indicated, digestions were stopped by removing an aliquot to a tube on ice containing a 1/10 volume of 10 mg of phenylmethylsulfonyl fluoride per ml. Digestion products were separated by SDS-PAGE, and immunoblot analysis was performed as described above.

Immunofluorescence. Polyclonal antibodies against Ssi1p were affinity purified on a column prepared by covalently attaching the glutathione *S*-transferase–Ssi1p fusion protein used to raise the antibodies (see above) to Affi-Gel 15 (Bio-Rad) according to the manufacturer's instructions. For immunofluorescence, cells were fixed for 2 h in 5% formaldehyde, spheroplasted with yeast lytic enzyme in 1.2 M sorbitol–0.1 M potassium phosphate (pH 6.5)–1% β-mercaptoethanol for 20 min at 30°C, washed twice with 1.2 M sorbitol–0.1 M potassium phosphate (pH 6.5), and fixed to polyethyleneimine-treated slides. Cells were blocked with 1% bovine serum albumin (BSA)–0.05% Nonidet P-40 for 5 min before overnight incubation with primary antibody in a moist chamber. After primary incubation, slides were washed extensively in PBS (pH 7.2)–0.05% Nonidet P-40, blocked again for 5 min with 1% BSA–0.05% Nonidet P-40, and incubated with fluorescein-labeled anti-rabbit secondary antibody (Boehringer Mannheim) for 1 h. Slides were again washed extensively before a 5-min incubation with 1 μg of 4,6-diamidino-2-phenylindole (DAPI) per ml. Finally, slides were rinsed in PBS and a drop of 0.1% phenylenediamine was added to each well before coverslips were applied and sealed in place. Cells were observed and photographed with an epifluorescence microscope coupled to a 35-mm camera (Nikon Inc., Melville, N.Y.).

Northern (RNA) analysis. Cells were grown to mid-log phase in YPD at 23°C and then treated for 1 h with 1 μg of tunicamycin per ml at 23°C or transferred to prewarmed YPD at 37°C and incubated for 30 min or 2 h. Ten milliliters of each culture was harvested, and cell pellets were frozen at −80°C. Total cellular RNA was isolated by the heat-freeze extraction method previously described (59). Total RNA fractions were enriched for poly(A)-containing RNA by using the PolyATtract mRNA Isolation System (Promega Corp.) according to the manufacturer's instructions. Enriched RNA fractions were separated on a 1% agarose–5.5% formaldehyde gel and transferred to a GeneScreen membrane (DuPont Co., Boston, Mass.) by capillary transfer. Probes were labeled by random priming, and hybridizations were carried out at 42°C overnight in hybridization buffer (50% formamide, 10% dextran, 5× Denhardt's solution, 1% SDS, 2.5 mM sodium PP_i, 50 mM Tris [pH 7.5], 1 M NaCl). The results were visualized by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). For reprobing, the membrane was first stripped by repeated washing with a boiling solution of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS, allowed to air dry, and incubated at 42°C with hybridization buffer for at least 6 h before the new probe was added.

Immunoprecipitation. Strains BB353 (wild type), BB355 (Δ*ssi1*), and RSY529 (*sec62*) were grown overnight at 30°C to mid-log phase in minimal medium containing 5% glucose. For proteinase A immunoprecipitations, cells were harvested and resuspended in the same medium at 4 units of optical density at 600 nm per ml, 20 μg of tunicamycin per ml was added, and cells were incubated for 15 min at 30°C prior to the addition of label. For labeling, cells were harvested, washed once in fresh minimal medium lacking methionine and cysteine (and with or without tunicamycin), and resuspended in the same medium at 4 to 10 units of optical density at 600 nm per ml. Approximately 150 μCi of protein labeling mix (DuPont) was added for each time point. After 10 min, labeling was stopped by the addition of unlabeled cysteine and methionine to 0.01%. At the time

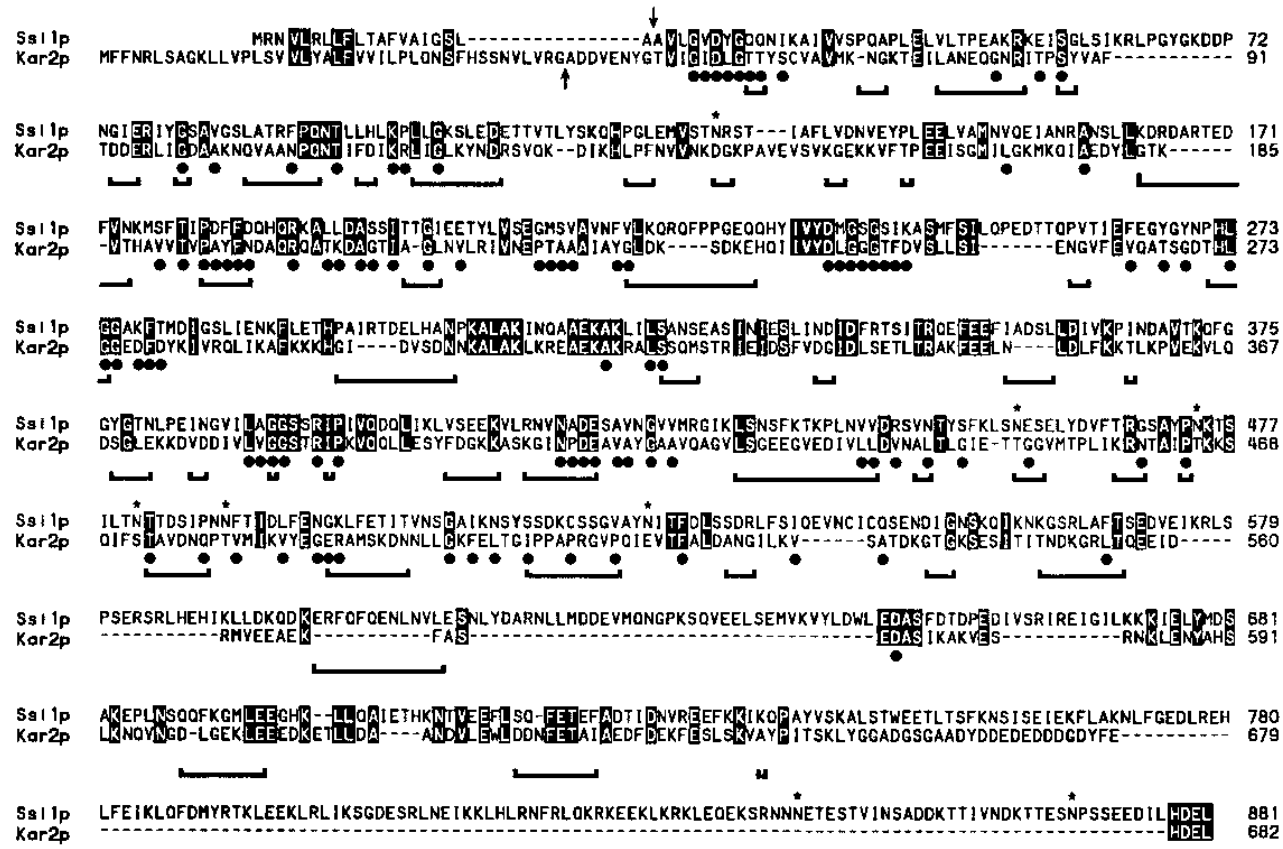

FIG. 1. Ssi1p shares 24% of the amino acids of Kar2p. The putative open reading frame of Ssi1p was aligned with Kar2p by using a Clustal alignment and the DNA Star software package. Amino acids shared by the two proteins are boxed. Residues that are shared by Kar2p and 35 other Hsp70s from various species (10) are indicated by solid circles below the Kar2p sequence. Each potential N-linked glycosylation site of Ssi1p (3) is marked by an asterisk. The signal sequence cleavage site of Kar2p (70), as well as the predicted cleavage site of Ssi1p (72), is depicted by an arrow. Loops and turns of the Kar2p tertiary structure, as predicted by analogy with the N-terminal domain of bovine Hsc70 (21, 22) and the C-terminal domain of DnaK (74), are indicated by brackets below the Kar2p sequence.

points indicated, aliquots of cells were harvested in 10 mM sodium azide, washed once in 1 mM sodium azide, resuspended in 100 μl of 1× SDS lysis buffer (1% SDS, 45 mM HEPES [pH 7.5], 15 mM DTT, 625 μg of phenylmethylsulfonyl fluoride per ml, and 125 μg of pepstatin per ml), and boiled. SDS lysates were diluted to 1 ml with Triton lysis buffer (150 mM NaCl, 50 mM HEPES [pH 7.5], 5 mM EDTA, 1% Triton X-100) and spun at top speed in a microcentrifuge at 4°C for 15 min. The supernatants were precleared for at least 30 min at 4°C with protein A-Sepharose (Sigma) and then immunoprecipitated overnight at 0°C with antibodies against either CPY or proteinase A (gifts from S. Emr and T. Stevens, respectively). Immunoprecipitates were isolated by the addition of protein A-Sepharose and another 30-min incubation at 4°C. Sepharose beads were washed three times with immunoprecipitation wash buffer (Triton lysis buffer plus 0.1% SDS), resuspended in 15 μl of 2× Laemmli sample buffer, and boiled. Immunoprecipitates were separated by SDS-PAGE and visualized with a PhosphorImager (Molecular Dynamics) or by autoradiography.

RESULTS

Analysis of the *SSI1* sequence. We identified *SSI1* in a search of the yeast sequence database for sequences with homology to the cytosolic Hsp70 Ssa1p. The *SSI1* sequence has an open reading frame of 2,643 nucleotides, potentially encoding a protein consisting of 881 amino acids, for a predicted size of 99.6 kDa. The predicted Ssi1p protein has similar levels of identity (approximately 25%) with yeast Hsp70s of various families, including Ssa1p, Ssb1p, and Sse1p of the cytosol, Ssc1p of the mitochondria, and Kar2p of the ER. This similarity includes the presence in Ssi1p of 40 of 101 amino acids conserved in all 36 Hsp70s of various species included in an early evolutionary analysis of the family (10); these 101 residues of Kar2p are indicated by filled circles in the alignment shown in Fig. 1. Sse1p of the cytosol, which was not included in the evolutionary analysis and represents the most distantly related Hsp70 family of *S. cerevisiae* reported to date, contains 48 of these residues. The sequence alignment of the predicted Ssi1p with Kar2p required the introduction of six gaps of at least four amino acids each in the N-terminal two-thirds of the Kar2p sequence (i.e., the 44-kDa ATPase domain, corresponding to the region from Ile-53 to Val-423). A comparison of Kar2p with the known structure of the bovine Hsc70 ATPase domain (21, 22) revealed that all of these gaps are within one residue of turn or loop regions in the structure, suggesting that this region is structurally conserved in Ssi1p (Fig. 1). The C-terminal portion (corresponding to the peptide binding domain of Kar2p) is less highly conserved in Ssi1p, which is the pattern for the Hsp70s in general. The structure of this domain in the *E. coli* Hsp70 DnaK has recently been determined (74); the loops and turns in the C terminus of Kar2p, as predicted from this structure, are shown in Fig. 1 (this structure corresponds to the portion of Kar2p from Gly-426 to the carboxy terminus). The predicted Ssi1p protein is approximately 25 to 30 kDa larger than a typical Hsp70; this additional sequence is primarily at the C terminus.

Analysis of the codon usage of *SSI1* by using the codon adaptation index (CAI) (62) gave a CAI of 0.147, as opposed to a CAI of 0.439 for *KAR2*. The CAI represents a comparison of actual codon usage in a particular gene to codon usage by a

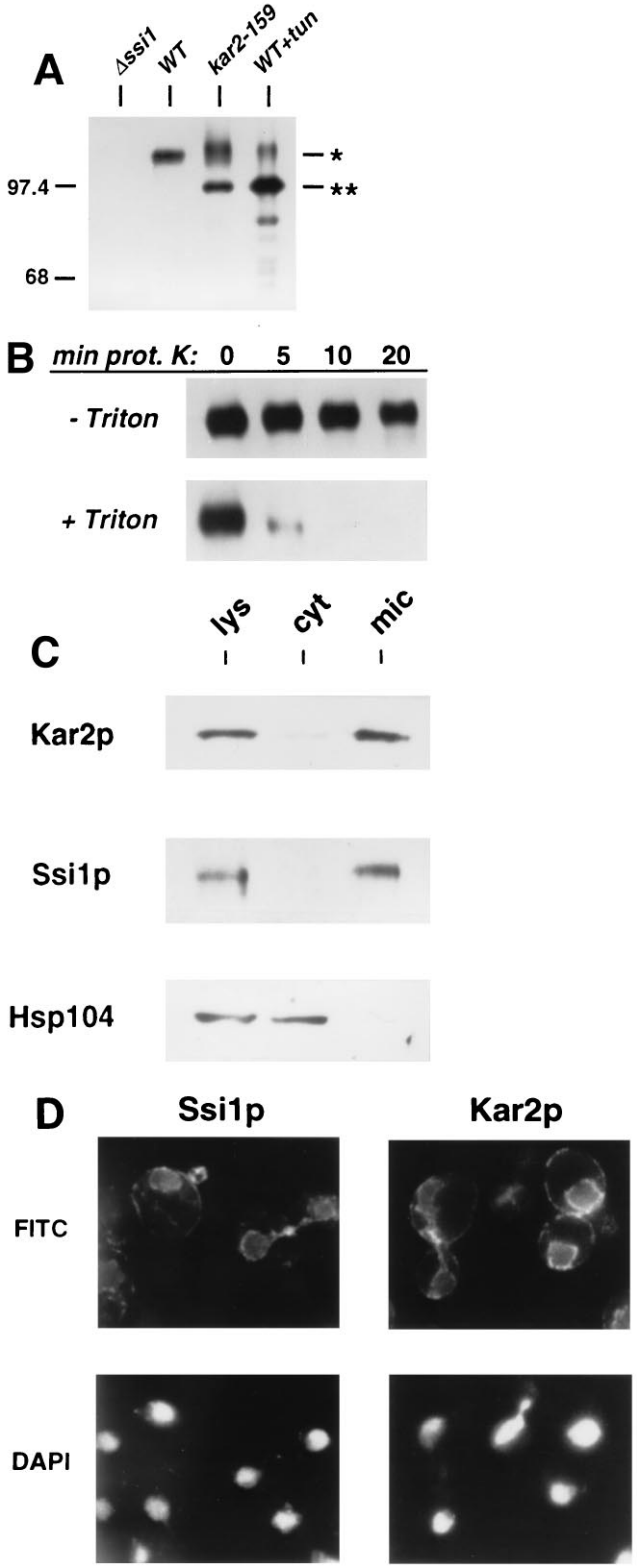

FIG. 2. Localization of Ssi1p to the ER. (A) Ssi1p immunoblot analysis. Strains were grown to mid-log phase at 23°C and shifted to 37°C with or without treatment with 10 μg of tunicamycin (tun) per ml for 2 h before harvest. Proteins were separated by SDS-PAGE in a 6% polyacrylamide gel, blotted to nitrocellulose, probed with polyclonal antibodies raised to Ssi1p, and visualized by enhanced chemiluminescence. The apparent positions of wild-type Ssi1p (*) and unglycosylated Ssi1p (**) are shown on the right. Molecular standards (in kilodaltons) are shown on the left. WT, wild type. (B) Protease protection assay. A homogenate of wild-type strain BB259 was treated with 25 μg of proteinase K (prot. K) per ml for the periods indicated in the absence (−) or presence (+) of 0.5% Triton X-100. Immunoblot analysis was performed as described for panel A. (C) Subcellular fractionation. A cleared lysate (lys) of wild-type strain BB259 was prepared, and soluble cytosolic (cyt) and microsomal (mic) fractions were derived from this lysate as described in Materials and Methods. Proteins were separated by SDS-PAGE in 7.5% (Kar2p) or 6% (Ssi1p and Hsp104) polyacrylamide gels, and immunoblot analysis was performed as described for panel A with antibodies to Kar2p, Ssi1p, or Hsp104. (D) Immunofluorescence. Affinity-purified antibody against Ssi1p showed perinuclear staining (top left) very similar to that seen with antibody against Kar2p (top right). Immunostaining was visualized with secondary antibodies conjugated to fluorescein isothiocyanate (FITC); DAPI was used to visualize nuclei (bottom). For Ssi1p immunofluorescence, wild-type strain BB259 was first transformed with a multicopy plasmid carrying *SSI1*. Staining was variable from cell to cell, presumably reflecting differences in the copy number of this plasmid. No staining was observed with the Ssi1p antibody in a Δ*ssi1* strain.

set of very highly expressed genes of *S. cerevisiae* and can vary from 0 to 1; highly expressed genes, such as those coding for ribosomal proteins, have CAI values from 0.5 to 1.0, while poorly expressed genes, such as *GAL4* and *PPR1*, have much lower CAI values, in the range of 0.1 (62). These data suggest that *SSI1* is likely to be expressed at significantly lower levels than is *KAR2*.

Several features of the predicted Ssi1p sequence are worthy of note. First, the four C-terminal amino acids are HDEL. This C-terminal sequence, which is shared by Kar2p, has been shown to function as an ER retention signal in *S. cerevisiae*, stimulating the retrieval of HDEL-bearing proteins from the Golgi apparatus back to the ER (48). Second, inspection of the Ssi1p sequence revealed a positively charged N terminus followed by a largely hydrophobic stretch extending from Leu-7 to Val-25; both characteristics suggest an ER signal sequence. Application of the algorithm of von Heijne (72) led us to predict a signal sequence cleavage site (indicated by an arrow in Fig. 1) after Ala-20. Third, the predicted Ssi1p amino acid sequence contains eight potential sites for N-linked glycosylation (N-X-S/T) (3), in contrast to Kar2p, which is completely devoid of such sites.

Ssi1p is localized to the ER. The presence of a yeast ER retention signal at the carboxyl terminus of Ssi1p, as well as a potential signal sequence at the N terminus, suggested to us that Ssi1p is localized to the ER. To directly address the localization of Ssi1p, we generated antibodies against a fusion construct between glutathione *S*-transferase and amino acids 513 to 835 of Ssi1p (see Materials and Methods). The specificity of this antibody preparation was demonstrated by the presence of a single major immunoreactive band in a wild-type strain grown under normal conditions and by the absence of an immunoreactive band in an *SSI1* deletion (Δ*ssi1*) strain (Fig. 2A, lanes 1 and 2). The wild-type immunoreactive band migrated more slowly than the expected size of either the full-length Ssi1p or its predicted cleavage product, which are 99.6 and 97.3 kDa, respectively. We hypothesized that this decreased migration was caused by modification of Ssi1p at one or more of its potential glycosylation sites (Fig. 1). To investigate this possibility, we treated a wild-type culture with tunicamycin, which inhibits N-linked glycosylation in the ER. This treatment resulted in the accumulation of a prominent, faster-migrating band which corresponded to the predicted size (Fig. 2A, lane 4), as well as several smaller fragments which presumably represent degradation products. A band which comigrated with the predominant band seen after tunicamycin treatment was seen in a *kar2-159* strain incubated at nonpermissive temperature (Fig. 2A, lane 3); this strain has previously been shown to manifest a temperature-dependent block in ER

translocation (71). The observed change in migration of Ssi1p upon either treatment of cells with tunicamycin or incubation of a translocation-deficient strain at its nonpermissive temperature suggests that the primary translation product of Ssi1p is normally translocated into the ER, where it is modified by glycosylation. No difference in migration between the Ssi1p precursor which accumulated in the *kar2-159* strain and the unglycosylated form seen in the presence of tunicamycin was observed, although we would have expected the latter to enter the ER and have its signal sequence removed. This observation is perhaps not surprising since the predicted sizes of Ssi1p with and without its signal sequence are 99.6 and 97.3 kDa, respectively, a difference which may well be below the limit of detection.

The suggestion from sequence and immunoblot analyses that Ssi1p resides in the ER was further supported by protease protection assays. To assess protease protection, homogenates were prepared from a wild-type strain and subjected to digestion by proteinase K. As would be expected for a protein of the ER lumen, immunoblot analysis demonstrated that Ssi1p was resistant to digestion unless detergent was added to disrupt intracellular membranes (Fig. 2B).

Direct evidence for the presence of Ssi1p in the ER was obtained from cell fractionation and immunofluorescence experiments (see Materials and Methods). In fractionation experiments, Ssi1p segregated with Kar2p to an enriched microsomal fraction, while Hsp104p, a soluble protein of the cytosol, segregated to a soluble fraction (Fig. 2C). Immunofluorescence experiments using affinity-purified Ssi1p-specific antibodies revealed perinuclear staining typical of resident ER proteins and very similar to that seen with antibodies raised against Kar2p (Fig. 2D).

***SSI1* is induced by tunicamycin.** Inspection of the *SSI1* promoter revealed a region with a high degree of similarity to the unfolded protein response element (UPR) of *KAR2* and a set of other genes which encode ER-localized putative chaperones (Fig. 3A). This element, which has been most extensively studied for its role in the regulation of *KAR2* (33, 41), is responsible for the induction of genes under conditions, such as tunicamycin treatment, which lead to the accumulation of unfolded proteins in the ER. Quantitation of our immunoblot analysis (Fig. 2A) by densitometric scanning, when normalized for total protein loading by Coomassie blue staining of a parallel gel, revealed that a 2-h treatment with 10 μg of tunicamycin per ml resulted in an elevation of Ssi1p protein levels of approximately twofold, as well as the change in electrophoretic mobility discussed above. In nine repetitions of this experiment, tunicamycin induction of Ssi1 protein was always seen; it ranged from 1.2- to 4.4-fold, with an average induction of 2.5-fold (data not shown). Northern analysis of wild-type cells given a milder tunicamycin treatment also demonstrated induction of *SSI1*, an effect that was quantitatively similar to the induction of *KAR2* under the same conditions (Fig. 3B, lanes 2 and 3). Incubation of a *kar2-159* mutant strain at the nonpermissive temperature, which should result in the accumulation of aberrantly folded ER proteins, produced a similarly high level of both messages (Fig. 3B; compare lanes 5 and 6). This response has been noted previously for *KAR2* (61) and is mediated through its UPR. Interestingly, although *KAR2* also contains a heat shock element in its promoter and is induced by a 30-min heat shock (45, 51), *SSI1* contains no such element and is not heat shock induced (Fig. 3B, lanes 3 and 4).

Disruption of *SSI1* results in moderate cold sensitivity and enhanced resistance to manganese. In order to assess the physiological role of *SSI1*, we created a construct which replaced a 774-bp *Pst*I-*Stu*I fragment of *SSI1* with the *LEU2* marker (Fig. 4A). A linear fragment containing this construct, which removes the first 244 amino acids of Ssi1p as well as 42 bp of 5′ noncoding region, was transformed into diploid yeast strain PJ53, and the integration of the *SSI1* disruption into the chromosome was confirmed by Southern blotting (data not shown). After sporulation of heterozygous diploids, asci were dissected and the progeny were analyzed for growth by using a variety of media and growth temperatures. Δ*ssi1* haploid strains in this background were viable, and their growth was similar to that of wild-type strains under most conditions. However, a slight cold sensitivity was consistently observed at temperatures below 30°C (Fig. 4B). Enhanced resistance to toxic levels of manganese was also observed, even at low temperatures at which Δ*ssi1* strains normally grow more slowly than do wild-type strains (Fig. 4B). No other differences were noted in tests of growth on glucose, glycerol, or galactose-based media or in the presence of a range of chemicals, including ethanol, hydrogen

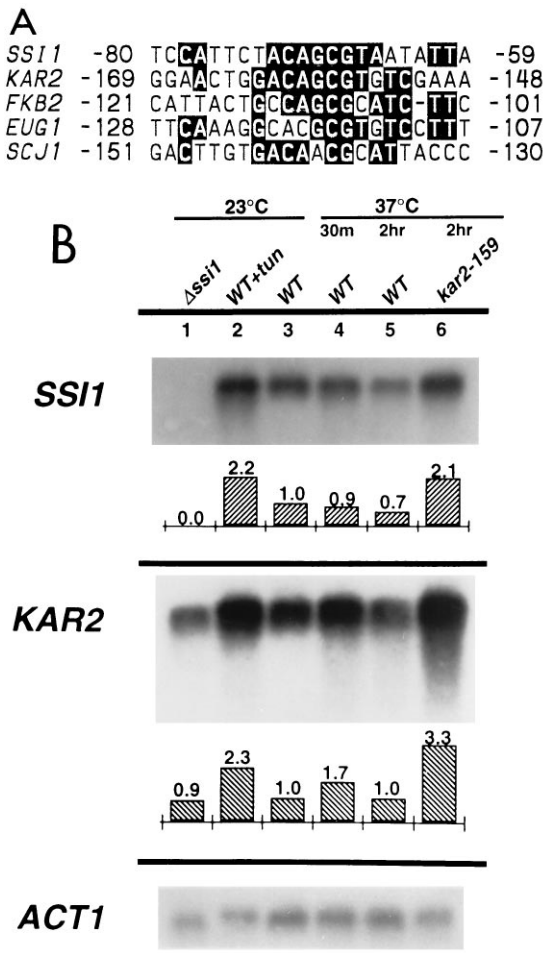

FIG. 3. Transcriptional regulation of *SSI1*. (A) Comparison of the putative UPR of *SSI1* with those of *KAR2*, *FKB2*, *EUG1*, and *SCJ1*. Nucleotides shared by at least three of the four sequences are boxed. Numbers refer to the location of each UPR in its promoter, relative to the translational start site at +1. (B) Northern analysis. Strains BB259 (wild type [WT]), BB258 (Δ*ssi1*), and BB316 (*kar2-159*) were grown to mid-log phase at 23°C and either received no further treatment (lanes 1 and 3), were treated for 1 h with 1 μg of tunicamycin (tun) per ml (lane 2), or were shifted to prewarmed flasks at 37°C for the periods shown (lanes 4 to 6). Northern analyses of poly(A)-enriched RNA preparations were performed as described in Materials and Methods. The data for *SSI1* and *KAR2* were quantitated with a PhosphorImager and standardized to an *ACT1* probing of the same blot; the results were normalized to those of the 23°C wild-type culture (lane 3) in each case and are presented graphically beneath each blot.

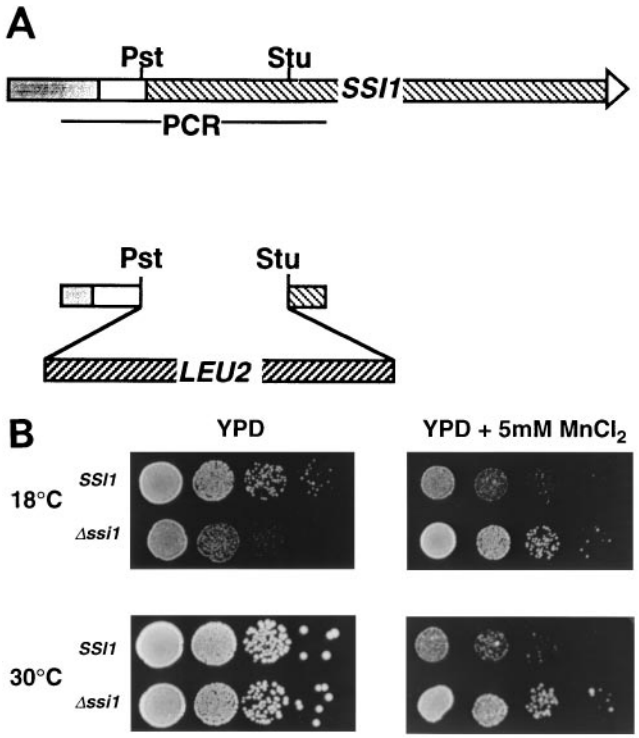

FIG. 4. Deletion of *SSI1* results in slight cold sensitivity and enhanced resistance to manganese. (A) Deletion-disruption strategy for *SSI1*. A 1.5-kb PCR fragment which contained the N-terminal 1.1 kb of *SSI1* was generated. This fragment was cloned into a vector and disrupted by insertion of the *LEU2* marker at the *Pst*I and *Stu*I sites. A linear fragment containing this disruption was used for transformation of diploid yeast cells. (B) Growth tests of wild-type strain BB259 and Δ*ssi1* strain BB258. Cells were counted in a hemocytometer, and equal numbers were spotted as a series of 10-fold serial dilutions to YPD plates supplemented with 0.004% adenine (to prevent the accumulation of red pigment in *ade2* strains), with (+) or without 5 mM manganese chloride. YPD plates were incubated for 2 days at 30°C or 3 days at 18°C; $MnCl_2$ plates were incubated for 3 days at 30°C or 4 days at 18°C.

peroxide, calcium, magnesium, tunicamycin, and β-mercaptoethanol (data not shown).

***SSI1* shows genetic interactions with *KAR2*.** Given the localization of Ssi1p to the ER and its homology to *KAR2*, we were interested to determine whether *SSI1* would show genetic interactions with genes encoding proteins involved in the secretory pathway. This approach has been used previously to demonstrate cooperativity among various components of the secretory pathway, including Kar2p and the DnaJ homologs of the ER, Sec63p and Scj1p (54, 58, 61). To this end, Δ*ssi1* strains were crossed with haploid strains carrying the conditional mutation *sec12-1*, *sec13-1*, *sec16-2*, *sec18-1*, *sec20-1*, *sec21-1*, *sec23-1*, *sec61-2*, *sec62*, or *sec63-1* to obtain diploids heterozygous at both *SSI1* and the respective *sec* locus. Then these diploids were sporulated, and the resulting tetrads were dissected. In all cases, viable spores which were both Leu^+ and temperature sensitive for growth were isolated, indicating they carried both Δ*ssi1* and the conditional *sec* allele. Deletion of *SSI1* is therefore not synthetically lethal with any of these secretory pathway mutations.

However, similar analyses of various conditional alleles of *KAR2* did reveal synthetic interactions with Δ*ssi1*. Dissections of tetrads from diploids which were heterozygous for deletion of *SSI1* and for the temperature-sensitive allele *kar2-159* consistently failed to yield any viable spores which were both Leu^+ and temperature sensitive for growth. This was true whether dissection plates were incubated at 30°C (35 tetrads), 23°C (36 tetrads), or 13°C (20 tetrads). Similar results were obtained with another temperature-sensitive allele, *kar2-113*; no viable double-mutant spores were obtained at any temperature. Three other conditional alleles of *KAR2* were analyzed. One of these, *kar2-191*, had no apparent synthetic growth defect in combination with the *SSI1* deletion. The other two alleles, *kar2-1* and *kar2-133*, while not synthetically lethal with a deletion of *SSI1*, produced a smaller number of viable double-mutant spores than expected. For *kar2-1*, 18 viable double-mutant spores were obtained from dissections of 72 tetrads at temperatures ranging from 13 to 30°C, indicating 25% viability; for *kar2-133*, 32 double mutants were obtained from 64 tetrads, indicating 50% viability. The viabilities of spores of other genotypes in these dissections ranged from 73 to 96%. In addition, viable *kar2-1* Δ*ssi1* or *kar2-133* Δ*ssi1* double-mutant spores, when obtained, produced colonies which grew more slowly than did those of other genotypes.

Synthetic effects with the *KAR2* mutations are allele specific. To more carefully analyze the interactions between *SSI1* and *KAR2*, *kar2* haploid segregants were first isolated from each heterozygous diploid and backcrossed three times to Δ*ssi1* strains to minimize the effects of differing genetic backgrounds. Heterozygous diploids from the final cross in each case were then transformed with a *URA3*-marked centromeric plasmid carrying a wild-type copy of *SSI1*. The diploid transformants were sporulated, and the resulting tetrads were dissected and analyzed. As expected from the dissection results reported above, all of the double mutants obtained from *kar2-113* or *kar2-159* heterozygous diploids carried the *SSI1* plasmid, while double mutants from *kar2-1*, *kar2-133*, or *kar2-191* heterozygous diploids were a mixture of Ura^+ and Ura^- isolates. Plasmid-bearing Δ*ssi1* strains carrying each of the five conditional *kar2* alleles were tested for the ability to grow on plates containing 5′-FOA, which is toxic to cells expressing the *URA3* gene product. In most strains, normal rates of plasmid missegregation result in a fraction of the population which has lost the plasmid and is therefore Ura^-; these segregants show growth on 5′-FOA. In the event that the *URA3*-based plasmid is itself required for growth, however, no portion of the population is able to grow. *kar2-113* Δ*ssi1* and *kar2-159* Δ*ssi1* strains were completely inviable on 5′-FOA at temperatures ranging from 18 to 38.5°C (Fig. 5A and data not shown), confirming that these strains required the plasmid-borne copy of *SSI1* for growth. Deletion of *SSI1* is therefore synthetically lethal with either *kar2-113* or *kar2-159*.

kar2-1 Δ*ssi1*, *kar2-133* Δ*ssi1*, and *kar2-191* Δ*ssi1* strains were all viable on 5′-FOA (Fig. 5A), indicating that they did not require *SSI1* for growth. Differences were observed, however, in the rates of growth of these strains at various temperatures. These differences were analyzed in more detail by examining serial dilutions over a range of temperatures of strains which had lost the *SSI1* plasmid. The results (Fig. 6) are distinctly different for each of the three *kar2* alleles.

Cells carrying a *kar2-133* mutation in the presence of a wild-type copy of *SSI1* were mildly temperature sensitive (Fig. 6). Deletion of *SSI1* in this *kar2* mutant background led to further impairment of growth at temperatures ranging from 18 to 34°C. In contrast, cells carrying the *kar2-191* mutation were severely impaired in growth across the range of temperatures from 18 to 34°C in the presence of a wild-type copy of *SSI1* and their growth was actually improved by *SSI1* deletion. *kar2-1* cells, which showed a mild temperature sensitivity similar to that of *kar2-133* cells in the presence of wild-type *SSI1*, showed a combination of effects upon *SSI1* deletion. In agreement with the tetrad dissection data reported above, *kar2-1* cells carrying

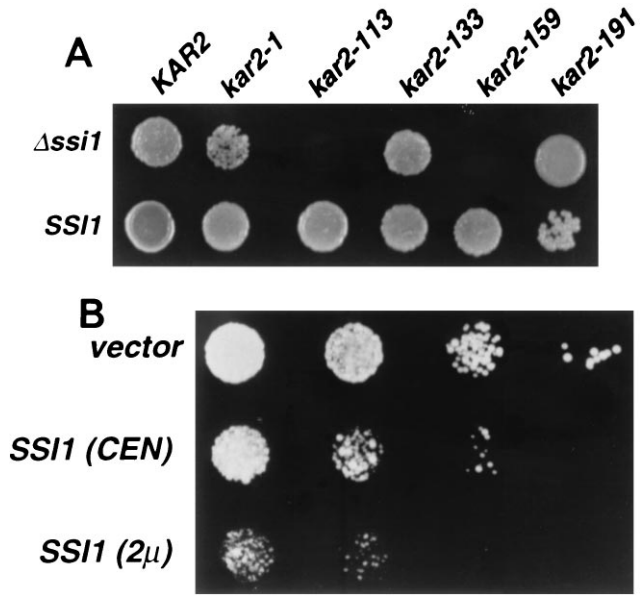

FIG. 5. (A) Synthetic lethality of *kar2-113* or *kar2-159* with deletion of *SSI1*. Δ*ssi1* strains possessing each of the indicated *KAR2* genotypes and carrying a centromeric plasmid containing *SSI1* were isolated and tested on 5′-FOA for the ability to grow without the plasmid. For comparison, wild-type *SSI1* strains of each *KAR2* genotype are shown as well. The 5′-FOA plate pictured was incubated at 23°C for 4 days. Similar results were obtained at 18 and 30°C and with an independent set of isolates at all three temperatures. (B) Extra copies of *SSI1* impair the growth of a *kar2-191* strain. *kar2-191* strain BB344 was transformed with pRS316 (vector), p316-SSI1 [*SSI1* (CEN)], or p426-SSI1 [*SSI1* (2μ)]. Transformants were grown overnight on selective media, counted, and spotted as 10-fold serial dilutions of equal numbers of cells. The selective plate shown was incubated at 23°C for 3 days; similar results were obtained at 18 and 30°C and with an independent set of isolates at all three temperatures. None of the transformants showed growth at a temperature of 34°C or above.

Δ*ssi1* grew significantly more poorly than did those containing *SSI1* at temperatures up to and including 30°C. However, at elevated temperatures (34 and 37°C), deletion of *SSI1* resulted in partial suppression of the temperature-sensitive growth of this strain, resulting in more robust growth at 34°C than is seen in any of the *kar2* single-mutant strains or even in the *kar2-191* Δ*ssi1* double mutant.

The allele specificity of these interactions did not correlate simply with the severity of the *kar2* mutations in terms of cellular growth, as *kar2-191* strains, like strains carrying *kar2-113* or *kar2-159*, showed a more severe temperature sensitivity than that of strains bearing either *kar2-1* or *kar2-133* (Fig. 6 and data not shown). The differences in the effects of deleting *SSI1* in various *kar2* mutant backgrounds may therefore indicate the cooperation of Ssi1p with Kar2p in vivo in a specific subset of cellular processes in which Kar2p is involved.

Additional copies of *SSI1* are deleterious for growth of a *kar2-191* mutant strain. In dissections of the various heterozygous diploids carrying the centromeric *SSI1* plasmid, we were able to isolate haploid strains of all genotypes which carried the plasmid with one exception: *kar2-191* isolates which had a wild-type genomic copy of *SSI1* were always Ura$^-$. Even when dissections were performed on plates lacking uracil to prevent the rapid loss of the plasmid, no Ura$^+$ *kar2-191* isolates were obtained. In combination with the results shown in Fig. 6 (deletion of *SSI1* partially suppressed the growth defect of *kar2-191* strains), this failure to obtain plasmid-bearing *kar2-191* isolates suggested to us that extra copies of *SSI1* might be deleterious in a *kar2-191* background. We tested this idea directly by transforming a *kar2-191* haploid strain with *SSI1* carried on either a centromeric or a multicopy vector. Transformants of each plasmid were obtained but showed impaired growth and viability when compared with those of the same strain transformed with a vector alone (Fig. 5B). As might be expected, this effect was more severe when *SSI1* was present on a multicopy plasmid rather than a centromeric plasmid.

Δ*ssi1* strains show a partial block in translocation of a variety of proteins into the ER. In conjunction with Sec63p and other factors, Kar2p plays a critical role in the translocation of proteins from the cytosol into the ER, and some conditional alleles of *KAR2* show a complete block in ER translocation at nonpermissive temperatures (for example, *kar2-113* and *kar2-159*; for reviews, see references 12, 56, and 71). Given the similarities of Ssi1p and Kar2p in terms of primary amino acid sequence and regulation and the genetic interactions of these two genes described above, we were interested to determine whether or not strains carrying a deletion of *SSI1* show an ER translocation defect.

In preliminary experiments, we observed an accumulation of a secretory precursor, prepro-α-factor, in Δ*ssi1* strains. The effect was mild, although reproducible, and no accumulation of cytosolic precursors of other secretory pathway proteins was detectable. This intriguing phenotype was thus difficult to study in detail. We have previously seen, however, that alterations of genes encoding molecular chaperones have distinct effects in the two strain backgrounds commonly in use in our laboratory (75). The *SSI1* experiments discussed so far were performed with strains derived from W303 (see Materials and Methods). In contrast, much of the published work from our laboratory regarding molecular chaperones and their regulation has been done with strains derived from DS110 (65), itself a derivative of S288C (8, 9, 28, 43, 44, 73). Accordingly, we created Δ*ssi1* strains in the DS110 background and analyzed ER translocation in these new strains.

In DS110-derived Δ*ssi1* strains, the accumulation of prepro-α-factor was readily apparent in *MAT*α cells, at dramatically higher levels than those seen for our original strains (Fig. 7A and data not shown). Although the antibody used in these experiments does not detect mature α-factor, its production by *MAT*α Δ*ssi1* strains of both backgrounds is demonstrated by their ability to mate normally, suggesting that the translocation of prepro-α-factor into the ER and its subsequent maturation and secretion are only partially impaired. Nonetheless, the readily detectable accumulation of this precursor in DS110-derived Δ*ssi1* strains led us to use these strains to examine the maturation of other proteins which transit the ER membrane.

Translocation of Kar2p into the ER results in the cleavage of its signal sequence, producing a detectable increase in migration of the protein on SDS-PAGE gels. Immunoblot analysis of Kar2p in wild-type strains generated a single band corresponding to mature Kar2p (Fig. 7B, lanes 1 and 3). In DS110-derived Δ*ssi1* strains, however, the cytosolic precursor of Kar2p was also readily apparent (Fig. 7B, lanes 2 and 4), indicating a partial block in translocation of Kar2p into the ER in these strains. Unlike mature Kar2p, pre-Kar2p was accessible to digestion by proteinase K in the absence of detergent, confirming its localization in the cytosol (Fig. 7C). Similarly, prepro-α-factor in these extracts was equally accessible to proteinase K in the absence and presence of detergent (data not shown).

To extend these results further, we used pulse-chase analysis to study the maturation of two vacuolar proteins, proteinase A (PrA) and CPY, in DS110-derived Δ*ssi1* strains. PrA maturation in the presence of tunicamycin was studied to enable us to distinguish its cytosolic and vacuolar forms; sorting of PrA is unaffected by these conditions (32). Prepro-PrA is cleaved in

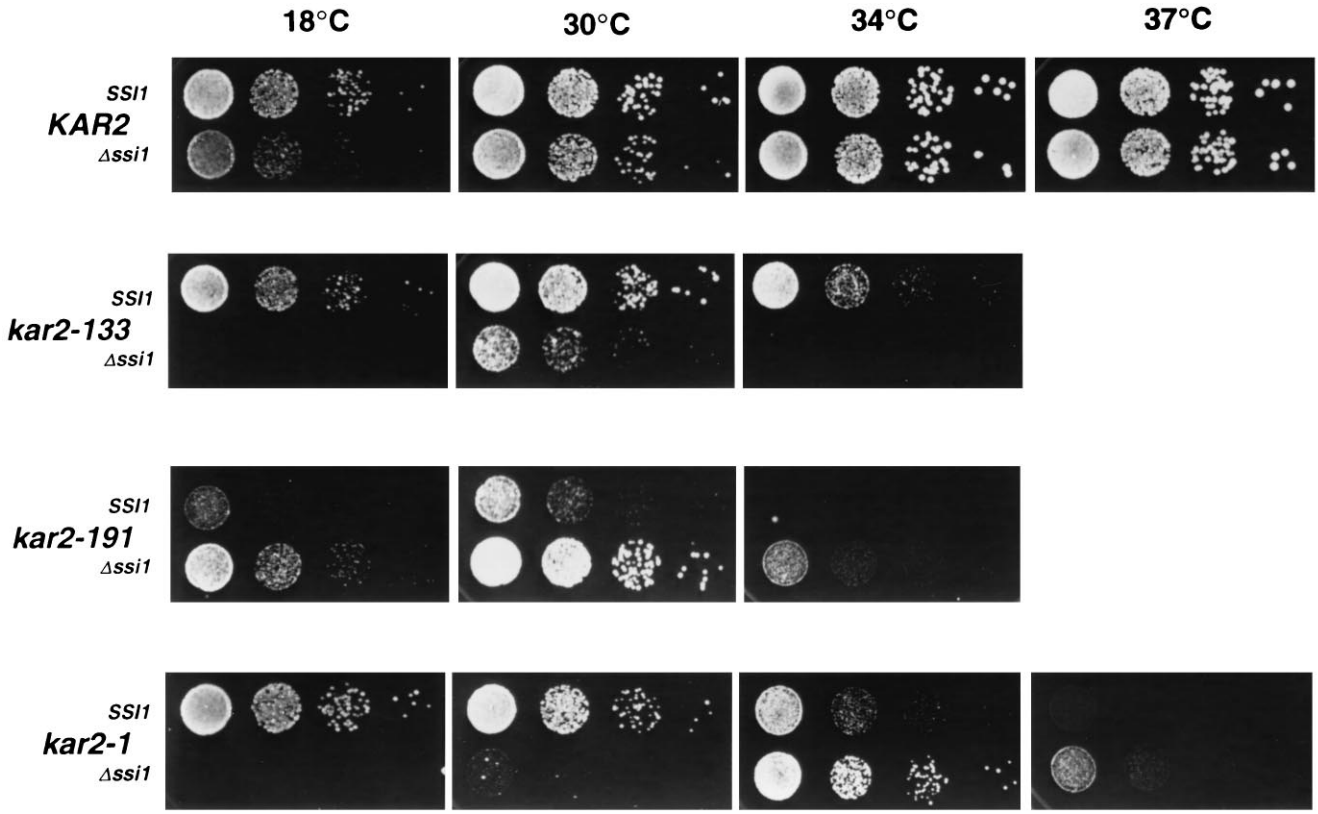

FIG. 6. Deletion of *SSI1* has different synthetic effects with different conditional *kar2* alleles. Δ*ssi1* strains of each of the indicated *kar2* genotypes which lacked the centromeric *SSI1* plasmid were selected by growth on plates containing 5′-FOA. Cells were counted in a hemocytometer, and equal numbers were spotted as 10-fold serial dilutions on YPD plates. Plates were incubated for 4 days at 18°C or 2 days at 30, 34, or 37°C. For comparison, *SSI1* wild-type strains of each *kar2* genotype are shown as well. Similar results were obtained with an independent set of isolates.

the ER to generate pro-PrA and then subsequently cleaved in the vacuole to generate the mature protein. In wild-type strains, the predominant form of PrA after a pulse is the ER form, which is processed to the mature vacuolar form over the course of the 15-min chase (Fig. 7D, lanes 1 to 3). In a Δ*ssi1* strain, processing of PrA was demonstrated by the appearance of the mature vacuolar form. However, the cytosolic prepro-PrA was also apparent throughout the chase (Fig. 7D, lanes 4 to 6); the identity of this band was confirmed by its comigration with the predominant species seen in the translocation-deficient *sec62* control strain (Fig. 7D, lanes 7 to 9).

An analysis of CPY maturation gave similar results. Immediately after pulse-labeling, wild-type strains showed predominantly the core-glycosylated P1 form and the fully glycosylated P2 form of CPY; after 20 min of chase, only the mature vacuolar form was evident (Fig. 7E, lanes 1 and 2). A Δ*ssi1* strain showed a similar pattern of maturation but also showed an accumulation of the cytosolic prepro-CPY, which was still evident after 20 min of chase (Fig. 7E, lanes 3 and 4). The accumulation of both prepro-PrA and prepro-CPY in Δ*ssi1* strains demonstrates a partial block in translocation of both precursors into the ER.

An analysis of the ER resident chaperone protein disulfide isomerase by immunoblotting also revealed an accumulation of its cytosolic form in Δ*ssi1* strains, as did pulse-chase analysis of the secreted protein invertase (data not shown). Thus, for every protein examined, α-factor, Kar2p, PrA, CPY, protein disulfide isomerase, and invertase, Δ*ssi1* strains demonstrated a partial defect in ER translocation.

The severity of the growth defect caused by deletion of *SSI1* varies with strain background. In addition to a more dramatic effect on protein translocation, deletion of *SSI1* in the DS110 background had a more severe effect on growth than it did in the W303 background. DS110-derived Δ*ssi1* cells showed significant growth impairment at the normal growth temperature of 30°C, conditions under which W303-derived Δ*ssi1* cells are indistinguishable from wild-type cells (Fig. 8). The distinction between the two backgrounds with regard to the effect of deletion of *SSI1* appears to be one of severity, with the general nature of the phenotype remaining the same. Like their W303-derived counterparts, DS110-derived Δ*ssi1* strains were cold sensitive, with the most severe impairment at the lowest temperature tested (18°C) and little to no defect at 37°C (Fig. 8). Δ*ssi1* strains in both backgrounds demonstrated enhanced resistance to manganese when compared with their *SSI1* counterparts (Fig. 4B and data not shown), and both demonstrate a partial block in translocation of at least one precursor protein, prepro-α-factor, into the ER. The reason for the difference in severity remains unclear.

DISCUSSION

Here, we have reported the characterization of a novel Hsp70 of the ER, Ssi1p. Strains carrying a deletion of *SSI1* are viable but show cold sensitivity and are unusually resistant to manganese. Among other functions, manganese is required for outer-chain glycosylation events in the Golgi (29, 46); manganese can also scavenge superoxide and hydroxyl radicals, and

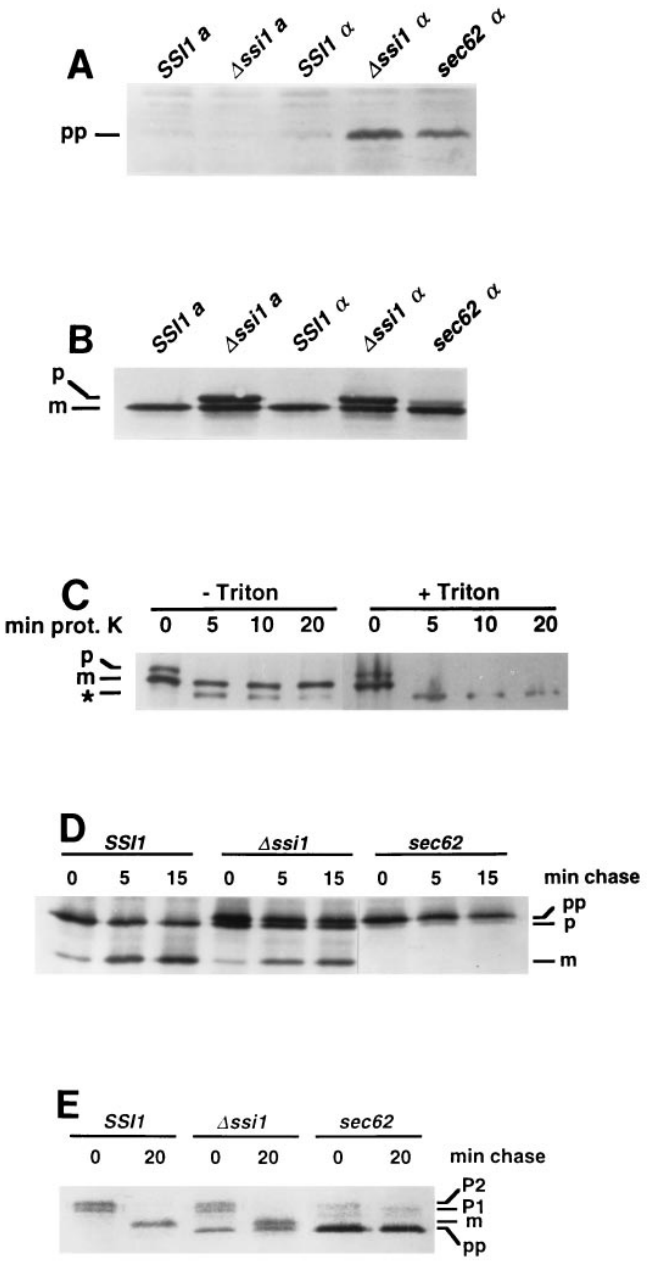

FIG. 7. Translocation of various precursors into the ER is impaired in Δ*ssi1* strains. (A) Immunoblot analysis of α-factor. Strains BB353 (*SSI1* **a**), BB355 (Δ*ssi1* **a**), BB344 (*SSI1* α), BB342 (Δ*ssi1* α), and RSY529 (*sec62* α) were grown to mid-log phase at 30°C and lysed as described in Materials and Methods. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, probed with antibody to prepro-α-factor (pp), and visualized by enhanced chemiluminescence. The antibody used (a gift of Jörg Becker) does not recognize mature α-factor, and significant steady-state levels of glycosylated pro-α-factor were not observed in this experiment. (B) Immunoblot analysis of Kar2p, performed as described for panel A. p, pre-Kar2p; m, mature Kar2p. (C) The Kar2p precursor (p) which accumulates in Δ*ssi1* strains is accessible to protease in the absence of detergent. A homogenate from Δ*ssi1* strain BB352 was prepared and treated with 10 μg of proteinase K (prot. K) per ml for the periods indicated in the presence (+) or absence (−) of Triton X-100 as described in Materials and Methods. Immunoblot analysis was performed as described for panel A. m, mature Kar2p; *, a relatively protease-resistant degradation product. (D) Immunoprecipitation of PrA. Strains BB353 (*SSI1*), BB355 (Δ*ssi1*), and RSY529 (*sec62*) were grown at 30°C, treated with tunicamycin, and labeled as described in Materials and Methods. The chase was initiated by the addition of unlabeled cysteine and methionine, and aliquots were removed at the indicated time points and lysed as described. Extracts were immunoprecipitated with antibody against PrA and visualized by autoradiography. pp, prepro-PrA (cytosolic form); p, pro-PrA (ER form; unglycosylated because of the presence of tunicamycin); m, mature PrA (vacuolar form). (E) Immunoprecipitation of CPY as described for panel C, except without tunicamycin treatment and visualized with a PhosphorImager. pp, prepro-CPY (cytosolic form); P1, core-glycosylated CPY (ER form); P2, fully glycosylated CPY (Golgi form); m, mature CPY (vacuolar form).

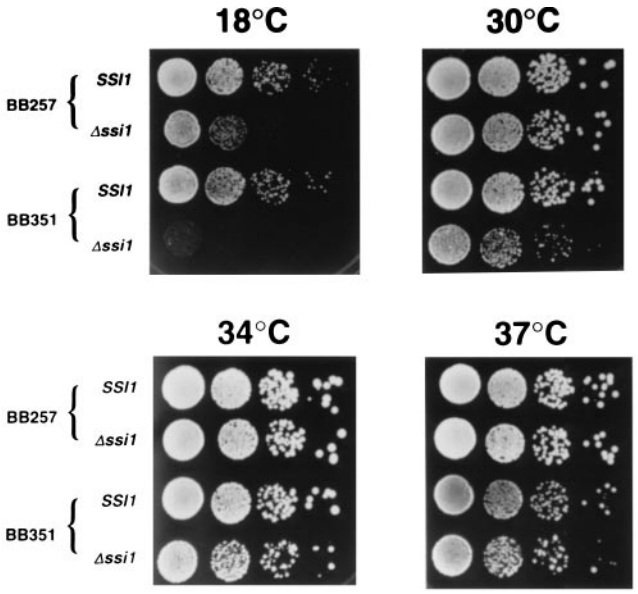

FIG. 8. The severity of the effect of *SSI1* deletion varies with strain background. Wild-type diploids BB257 and BB351 (see Materials and Methods) were transformed with a linear DNA fragment designed to disrupt *SSI1*; disruptions were confirmed by Southern analysis. Haploid segregants of the indicated genotype were obtained from each diploid and tested for growth on YPD plates (supplemented with 0.004% adenine to prevent the accumulation of red pigment in *ade2* strains) at various temperatures. Cells were counted in a hemocytometer, and equal numbers were spotted as 10-fold serial dilutions. Plates were incubated for 2 days at 30, 34, and 37°C and for 3 days at 18°C.

high levels of extracellular manganese can suppress the phenotypes of yeast strains missing copper/zinc superoxide dismutase (encoded by *SOD1*) (1, 4, 15, 34, 36, 68). The relevance of these effects to manganese toxicity is currently unclear, as are the implications of the observed manganese resistance of Δ*ssi1* strains.

We observed differences in the severity of the effects of deletion of *SSI1* between yeast strains derived from DS110 and those derived from W303. While deletion strains in both backgrounds showed similar resistance to manganese, both the cold sensitivity and the translocation defect were more marked in DS110-derived strains. The reason for this difference in severity remains unclear but will be an interesting area for further study.

Consistent with the presence in the *SSI1* promoter of an element with strong similarity to the unfolded protein response element of *KAR2*, expression of *SSI1* is induced by tunicamycin. Since the identification of the UPR element in the *KAR2* promoter (33, 41), similar elements have been identified in *FKB2*, which encodes a peptidyl-prolyl *cis-trans* isomerase (47); in *EUG1*, which encodes a homolog of protein disulfide isomerase (67); and in *SCJ1*, which encodes a homolog of the Hsp70-interacting protein from *E. coli*, DnaJ (58). Like *KAR2*, all these genes encode ER resident proteins and are induced by tunicamycin treatment.

The induction of these diverse genes by tunicamycin sug-

gests that their products play a role in protein folding. Fkb2p belongs to a family of proteins which accelerate folding by catalyzing the isomerization of *cis* and *trans* peptide bonds involving proline (47). Similarly, Eug1p is homologous to and can functionally substitute for protein disulfide isomerase, which catalyzes the isomerization of disulfide bonds in the ER (67). Kar2p is required for the proper folding and modification in the ER of at least one substrate, CPY (64); Scj1p may cooperate with Kar2p in this process. The identification of Ssi1p and its regulation by a putative UPR add another member to this rapidly growing group of resident ER proteins. The elucidation of the interrelationships of these various factors in protein folding in the ER requires further study.

While this paper was in review, the work of another group on the characterization of *SSI1*, which they call *LHS1*, was published (20). For the most part, their findings are corroborated and extended by this report; the phenotypes observed were most similar to those of our DS110-derived strains. Interestingly, they noted synthetic lethality between deletion of *SSI1* and an *ire1* null mutation. Because *IRE1* encodes a protein necessary for the unfolded protein response (17, 40), they suggest that UPR-mediated induction is necessary for survival in the absence of Ssi1p. This effect may be dependent on strain background, as we did not observe induction of *KAR2* mRNA in a W303-derived Δ*ssi1* strain (Fig. 3B).

In addition to protein folding, Kar2p is required for the translocation of cytosolic precursors into the ER. Upon upshift to nonpermissive temperatures, strains carrying one of several conditional *kar2* alleles, including *kar2-159* and *kar2-113*, manifest severe translocation blocks. These strains also rapidly lose viability after upshift (12, 13, 38, 56, 71). Characterizations of a large number of conditional alleles of *KAR2* have led to their division into three classes according to phenotype. *kar2-159* and *kar2-113* are representative class I alleles. Class II alleles, which include *kar2-1* and *kar2-133*, remain viable at nonpermissive temperatures, do not block translocation, and show a variety of pleiotropic defects in the processing of secreted proteins. Class III alleles, which include *kar2-191*, show a combination of class I and class II phenotypes, exhibiting posttranslocational defects analogous to those of class II but showing some translocation defects and loss of viability upon prolonged exposure to nonpermissive temperatures (50, 53). It is interesting that the genetic interactions of various *kar2* mutant alleles with a deletion of *SSI1* are divisible by *kar2* mutant class. Class I alleles show synthetic lethality in combination with Δ*ssi1*; class II alleles show impaired growth, at least at normal temperatures; and the one class III allele analyzed, *kar2-191*, actually shows improved growth in the presence of an *SSI1* deletion. In addition, *kar2-191* strains show impaired growth in the presence of extra, plasmid-borne copies of *SSI1.*

Genetic interactions with *KAR2* which are specific to certain *kar2* mutant alleles have been previously observed with other genes. *SCJ1* encodes a yeast DnaJ homolog which is localized to the ER and is not essential for growth. Reminiscent of our results with *SSI1*, deletion of *SCJ1* is synthetically lethal with the class I allele *kar2-159*, but not with the class II alleles *kar2-1* and *kar2-133* (58). *SEC63* encodes another DnaJ homolog of the ER and is essential for growth, but several conditional alleles have been isolated. An extensive analysis of the interactions between *sec63-1* and a variety of *kar2* alleles revealed synthetic lethality with some *kar2* mutations but not others. These interactions were not simply divisible by class, however. For example, the class II alleles *kar2-1* and *kar2-133*, as well as the class I allele *kar2-159* and the class III allele *kar2-191*, were synthetically lethal with *sec63-1*, but the class I allele *kar2-113* was not (61). Since Sec63p can be isolated in a complex with Kar2p (13), the explanation for the observed effects may be that Sec63p interacts physically with Kar2p and is thus particularly sensitive to the nature of the *kar2* mutation involved, while Scj1p and Ssi1p have overlapping functions with Kar2p (as represented by a particular class of *kar2* alleles) but do not necessarily physically interact with Kar2p.

Synthetic lethality of *SSI1* deletion with class I *kar2* alleles, as well as the partial translocation block that we observed in Δ*ssi1* strains, suggests that Ssi1p cooperates with Kar2p and other ER resident proteins in the translocation of cytosolic precursors into the ER. Ssi1p appears to be dispensable for this process under normal conditions, as evidenced by the production in Δ*ssi1* strains of some of the mature form of each protein we examined and by the viability of these strains. In the presence of a class I mutation such as *kar2-113* or *kar2-159*, however, Ssi1p becomes essential for growth. It is possible that Ssi1p is able to functionally substitute for Kar2p in the translocation process, at least to some extent. In this model, the *kar2-113* and *kar2-159* alleles produce proteins which are able to function well enough in translocation to allow growth at normal temperatures as long as Ssi1p is present, but the deletion of *SSI1* in these backgrounds is lethal. In contrast, the class II *kar2-133* allele produces protein which is not severely impaired in facilitating translocation; thus, Ssi1p is not essential in this background. An explanation of our results in the *kar2-191* background is more complicated. One possibility is that the protein produced by *kar2-191* (and perhaps, at high temperatures, the protein produced by *kar2-1*) interacts negatively with the translocation apparatus and that this interaction is facilitated by Ssi1p. In this situation, deletion of *SSI1* would reduce that negative interaction and thus actually be beneficial for growth, as we observed. The possibility that the protein encoded by *kar2-191* has altered activity is supported by the finding of Scidmore et al. that *kar2-191* is synthetically lethal with *sec63-1*, even in the presence of a wild-type copy of either gene (61).

An alternative explanation for our results is that Ssi1p is not directly involved in translocation but is important for a later step in protein trafficking through the ER, such as folding and modification. Interference with this later step through deletion of *SSI1* might result indirectly in the translocation defects observed. Our data do not support this model, as it appears from the pulse-chase analyses of PrA and, particularly, CPY that substrate molecules which do cross the ER membrane are processed and mature normally. However, it remains possible that there is a folding defect in Δ*ssi1* strains which is not detectable by the methods we used and that the resulting accumulation of misfolded proteins diverts Kar2p from translocation, leading to the observed translocation block. The exact functional role of Ssi1p thus awaits further analysis.

Until recently, the mitochondria and ER of *S. cerevisiae* were each thought to contain a single, essential member of the Hsp70 family, Ssc1p and Kar2p, respectively. Recently, a novel Hsp70 of mitochondria, Ssh1p, was identified. Deletion of *SSH1* results in marked cold sensitivity, but the cold sensitivity of Δ*ssh1* strains can be suppressed by overexpression of *SSC1*, which encodes the essential Hsp70 of the same compartment. This suggests that Ssc1p and Ssh1p functionally overlap (57). Like *SSI1*, *SSHI* is thought to be expressed at a much lower level than the essential Hsp70 of the same compartment, *SSC1*. The identification of *SSI1* demonstrates that mitochondria and the ER each contain two Hsp70s, one that is essential and highly expressed and another that is dispensable for growth under normal conditions, expressed at lower levels, and shows some functional overlap with the first.

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