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Sls1p, an Endoplasmic Reticulum Component, Is Involved in the Protein Translocation Process in the Yeast *Yarrowia lipolytica**

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Signal recognition particle-dependent targeting of secretory proteins to the endoplasmic reticulum membrane is predominant in the yeast *Yarrowia lipolytica*. A conditional lethal mutant of the *SCR2*-encoded 7S RNA provided the first *in vivo* evidence for involvement of this particle in cotranslational translocation (He, F., Beckerich, J. M., and Gaillardin, C. M. (1992) *J. Biol. Chem.* **267**, 1932–1937). In order to identify partners of 7S RNA or signal recognition particle in their function, we selected synthetic lethal mutations with the 7S RNA mutation (*sls*). The *SLS1* gene, cloned by complementation of the *sls1* mutant growth defect, encodes a 426-amino acid polypeptide containing a NH₂-terminal signal peptide and a COOH-terminal endoplasmic reticulum (ER) retention motif. The *SLS1* gene product behaves as a lumenal protein of the ER. Sls1p was sedimented with membrane-rich organelles and was resistant to protease degradation without prior membrane solubilization. Immunofluorescence microscopy showed a typical endoplasmic reticulum perinuclear staining. Co-immunoprecipitation revealed that Sls1p resides close to the major translocation apparatus component, Sec61p. Deletion of the *SLS1* gene led to a temperature-sensitive growth phenotype. Synthesis of several secretory proteins was shown to be specifically reduced in Δ*sls1* cells. We propose that Sls1p acts in the preprotein translocation process, interacting directly with translocating polypeptides to facilitate their transfer and/or help their folding in the ER.

In order to enter the secretion pathway, secretory proteins of eukaryotic cells have to be transported across or inserted into the endoplasmic reticulum (ER)[1] membrane. To achieve this translocation step, secretory proteins must be specifically targeted to the translocation machinery in the ER membrane and be competent for crossing this membrane (2). In higher eukaryotes, the signal recognition particle (SRP) was shown to take part into these functions (3). SRP is composed of a single 7S RNA and six polypeptides (4). When the signal sequence of a nascent secretory polypeptide is extruded from the ribosome,

it is first recognized by the nascent polypeptide associating complex (5), which allows specific binding of SRP. Interaction of SRP with the nascent chain-ribosome complex causes translational slow down. After binding of SRP to its membrane-bound receptor, SRP is displaced from the complex and the nascent chain is transferred to the translocation site where crossing takes place simultaneously to translation. As soon as the polypeptide emerges in the lumen of the ER, it interacts with various proteins for processing and folding. A somewhat different picture emerged from studies on the yeast *Saccharomyces cerevisiae*. Indeed, several secretory proteins in this yeast appeared to be transported post-translationally, both *in vivo* and *in vitro* (6, 7), and homologues of mammalian SRP components that have been identified in this yeast and function in translocation (8–11) are not essential for cell viability. In another yeast *Yarrowia lipolytica*, deletion of both genes *SCR1* and *SCR2* encoding 7S RNA is lethal (12), and we suggested earlier that the SRP-dependent targeting may be the main pathway, as in higher eukaryotic cells.

Isolation of conditional lethal mutants in the 7S RNA provided *in vivo* evidence for involvement of SRP in cotranslational translocation (1, 13). In order to identify partners of SRP in this process and to better understand its molecular mechanisms, we have now selected synthetic lethal mutations with the 7S RNA mutation, called *sls*. In the present paper, we describe identification and characterization of one of these genes, *SLS1*, and of its gene product.

MATERIALS AND METHODS

Strains, Growth Conditions, and Materials—For DNA manipulation, *Escherichia coli* strains TG1 or DH5α were used. *E. coli* cells were grown at 37 °C in LB medium (2.5% Luria broth base). Ampicillin was added at 100 μg/ml.

The *Y. lipolytica* haploid mutagenized strain was *MatB, scr1::ADE1*, Δ*scr2, ura3, leu2, his*-1, containing the replicative plasmid pINA1090 carrying the *scr2*-II.13 allele and the *URA3* gene. Replacement of the *scr2*-II.13 allele by the wild-type allele was done by plasmid shuffling using the replicative plasmid pINA237, which contained the *SCR2* gene and the *LEU2* gene. For genetic studies, the *sls1* Ts mutant was mated with a *MatA, scr1::ADE1*, Δ*scr2, lys11, ura3, leu2* strain containing the plasmid pINA398 carrying the *SCR2* gene and the *URA3* gene. Diploids were sporulated and analyzed as described previously (12). To isolate the *SLS1* gene from a *LEU2*-based replicative genomic library constructed by P. Fournier, a Leu⁻ Ts segregant from this cross was retained. Transformation of *Y. lipolytica* by the lithium acetate method was performed as described previously (14). *Y. lipolytica* strains were usually grown at 28 °C in YPD (1% yeast extract, 1% bacto-peptone, 1% glucose). For transformant selection, minimal medium (0.17% yeast nitrogen base without ammonium sulfate and without amino acids, 1% glucose, 0.1% proline) was used, and supplements were added to a final concentration of 0.01%. 5-Fluoroorotic medium contained 0.001% of uracil and 0.125% of 5-fluoroorotic acid. AEP induction was performed using GPP medium (2% glycerol, 0.17% yeast nitrogen base without ammonium sulfate and without amino acids, 0.3% proteose peptone, 50 mM phosphate buffer, pH 6.8, and appropriate supplements for the growth of cells). For labeling, GC medium (same as GPP but 0.2% casein instead of 0.3% proteose peptone) was used.

[1] The abbreviations used are: ER, endoplasmic reticulum; SRP, signal recognition particle; AEP, alkaline extracellular protease; kb, kilobase(s); bp, base pair(s); PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; Ts, temperature-sensitive.

Plasmids and Nucleic Acid Manipulations—E. coli plasmid pBS (Bluescript, from Stratagene) was used for DNA sequencing of a 2-kb *Cla*I-*Sal*I fragment carrying the *SLS1* gene. pINA237 and pINA398 containing the *SCR2* allele have been described previously (1). To test *sls1*-1 monocopy complementation, a 3.4-kb *Xba*I-*Hin*dIII fragment was cloned between the unique sites *Nhe*I and *Hin*dIII of an integrative plasmid. Integration was directed at the *LEU2* locus of the *sls1* Ts mutant after linearization with *Apa*I, which cuts in the *LEU2* gene. The *SLS1* inactivated copy was constructed in two steps: a 963-bp deletion of the *SLS1* coding region was first created by ligation of a 550-bp *Eco*RI-*Ssp*I fragment with a 5.8-kb *Pvu*II-*Eco*RI fragment, and a 1.65-kb *Sal*I-*Sal*I fragment containing the *URA3* gene was then cloned in the *Xho*I site 55-bp upstream the *Hin*dIII site. The 2.45-kb *Cla*I-*Hin*dIII fragment was directly used to transform a *MatB, scr1::ADE1, ura3, leu2, his*-1 strain (INAG 136463). A truncated copy of the *SLS1* gene was obtained after insertion of a polymerase chain reaction-amplified 1150-bp internal fragment in an integrative vector. Linearization of the recombinant plasmid by *Xmn*I, which cuts within the *SLS1* coding region directed integration at the *SLS1* locus of INAG 136463, creating a tandem repeat of 3′- and 5′-truncated copies of *SLS1*.

Anti-Sls1p Antibodies—To raise antibodies against the *SLS1* gene product, a large fragment of the *SLS1* coding region was fused in frame with the glutathione *S*-transferase-encoding gene from *Schistosoma japonicum* using the vector pGEX-2T (15). Nucleotides 61–1210 of *SLS1* were amplified by polymerase chain reaction using two primers. The first, flanked by a *Bam*HI site, was 5′-CGCGGATCCGATGAAATCTG-CAGAGTT-3′; the second one, in opposite orientation and flanked by an *Eco*RI site, was 5′-CCGGAATTCTAAGCTTGACGAGATCAT-3′. The polymerase chain reaction product was inserted as a *Bam*HI-*Eco*RI fragment in pGEX-2T. The recombinant 70-kDa protein was expressed at high levels in *E. coli* cells after a 4-h induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. After centrifugation, cells were resuspended in ice cold phosphate-buffered saline (PBS), 1% Triton X-100 and then lysed by sonication. Supernatant was passed over a glutathione-Sepharose column. After washes in PBS, the fusion protein was eluted with 5 mM glutathione in 50 mM Tris, pH 8. The hybrid protein was used to immunize subcutaneously rabbits after denaturation with 0.5% SDS at 80 °C and dilution in PBS. For the first injection, the protein was mixed with Freund's complete adjuvant. Two booster injections were done at intervals of 4 weeks using incomplete adjuvant. For each injection, 50 μg of the fusion protein was injected. Sera were collected after the last boost and tested by immunoblotting. They recognized a single product and were thus used at 1:1000 dilution.

Immunofluorescence—Cells grown in rich YPD medium to optical density of 1–2 were fixed by the addition of 5% formaldehyde to the culture. After centrifugation, cells were incubated for 2 h at room temperature without shaking in 50 mM potassium phosphate buffer, pH 6.5, 0.5 mM MgCl$_2$, 5% formaldehyde. Cells were then resuspended in 10 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 25 mM β-mercaptoethanol and permeabilized using 10 mg of Zymolyase 20T and 20 mg of Cytohelicase during 40 min at 37 °C. 10 μl of the cell suspension in PBS were put in wells of immunofluorescence slides pretreated with polylysine. Cells were treated with 10 μl of PBS plus 0.5% bovine serum albumin and 0.05% Nonidet P-40 during 15 min and washed before the addition of 10 μl of 1:300 diluted anti-Sls1p antibodies. After 1 h of incubation and washes in PBS, bound primary antibodies were decorated with 1:300 diluted fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. Slides were treated with 300 μg/ml 4,6-diamidino-2-phenylindole and mounted in one drop of mounting medium (1:10 PBS, 9:10 glycerol, 1 mg/ml *p*-phenylenediamine).

Membrane Fractionation and Cross-linking—25 ml of *Y. lipolytica* cultures grown overnight to early-log phase (optical density, 1–2) were centrifugated, and cells were resuspended in 500 μl of spheroplast buffer (1.5 M sorbitol, 50 mM Tris-HCl, pH 8, 2 mM MgCl$_2$, 60 mM β-mercaptoethanol) after washing. 130 units of zymolyase 20T were added per optical density unit of cells, and incubation was performed for 30 min at 30 °C. 500 μl of 1.8 M sorbitol were added, and protoplasts were pelleted after cooling and centrifugation. After resuspension in 200 μl of lysis buffer (0.2 M sorbitol, 0.1 M NaCl, 25 mM NaPi, pH 7.4, 1 mM MgCl$_2$, 1 mM phenylmethylsulfonyl fluoride), glass beads were added, and cells were lysed by 4 × 15-s vortexing. Clarified extracts were harvested after a 2-min centrifugation at 450 × *g*. Total protein content was measured by the Bradford assay (Bio-Rad S.A., Ivry-Sur-Seine). For membrane fractionation, extracts were further fractionated by a 20-min centrifugation at 10,000 × *g*. The supernatant and pellet were collected. For the protease protection experiment, the latter pellet was resuspended in 120 μl of lysis buffer and divided into three parts. Each was incubated for 1 h on ice; two were treated with 0.5 mg/ml of

proteinase K in the absence of any detergent or in the presence of 4% Triton X-100, and the last was left untreated. Reactions were stopped by the addition of 20% trichloroacetic acid. For cross-linking, 0.2 mg/ml dithiobis(succinimidyl propionate) were added to 200 μl of clarified extracts simultaneously to 1% Triton X-100. After a 20-min incubation at room temperature, the reaction was stopped by the addition of 0.2 M NH$_4$Ac and incubation on ice for 10 min. Samples were then incubated 10 min at 65 °C with 1% SDS before dilution with 0.8 ml of PBS and immunoprecipitation. Immunoprecipitation was performed at 4 °C during 3 h in the presence of antibodies and 10 μl of protein A-Sepharose. Extracts and PBS-washed immunoprecipitates were diluted with 2 × SDS-PAGE buffer containing 50 mM dithiothreitol and heated 10 min at 95 °C before loading on SDS-polyacrylamide gels. After migration, proteins were electrotransferred to nitrocellulose membranes (Schleicher & Schuell). Preincubation with 2% milk, antibody incubations, and washes were done in 10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20 buffer.

Pulse-Chase Labeling and Immunoprecipitation of AEP—Cells grown overnight in GPP medium at 18 °C were concentrated to 1,000 klett in GC medium and equilibrated at 18 °C for 20 min before a 1-h shift to a nonpermissive temperature of 28 °C. Cells were radiolabeled with 750 μCi of L-[4,5-^{3}H]leucine for 45 s and then chased with a 300-fold excess of cold L-leucine. 3-ml aliquots were withdrawn at 0, 1, 2.5, and 5 min and mixed with 2.3 g of crushed ice plus 10 mM NaN$_3$ and 2 mM phenylmethylsulfonyl fluoride to stop protein synthesis. After centrifugation, supernatants were precipitated with 10% trichloroacetic acid on ice for 1 h. Precipitates were dissolved in 200 μl of SDS-PAGE loading buffer and neutralized with 1 M Tris, pH 9.5. Cell extracts, prepared as described previously (16), were adjusted to 1% SDS and 4% Triton X-100 before immunoprecipitation. Total incorporation of label was measured at two post-chase time points by scintillation counting of boiled trichloroacetic acid precipitates from two 100-μl samples of labeled cell suspension. Proteins were analyzed by electrophoresis in 10% polyacrylamide gels, after which they were dried and fluorographed.

Glycoprotein Precipitation with Concanavalin A-Sepharose—Cells were grown in minimal medium at 20 °C. 25 optical density units of cells were resuspended in 0.8 ml of sulfate-free synthetic medium and incubated for 30 min before labeling with 500 μCi of Tran^{35}S-label for 10 min at 26 °C. Cells were chased with 200 μl of 50 mM cold methionine and cysteine and 10 μl of 1 M NaN$_3$. 100 μl of the 200 μl of clarified extract was diluted with 400 μl of concanavalin A buffer (15 mM NaPi, pH 7, 150 mM NaCl, 1% Triton X-100, 0.1% SDS), and 40 μl of packed concanavalin A-Sepharose beads was added, followed by incubation at room temperature for 2 h. After four washes, bound proteins were dissociated in SDS-PAGE buffer by heating at 95 °C for 5 min. Glycosylated proteins were analyzed by electrophoresis in 6% polyacrylamide gels. Gels were then dried and fluorographed.

RESULTS

Isolation of a Mutant That Displays Synthetic Lethality with the scr2-II.13 Mutant—To identify partners of the 7S RNA and/or the SRP in the cotranslational translocation process, we looked for new mutations aggravating the phenotype of the conditional *scr2*-II.13 mutation. Previous studies have indicated that such synthetic lethal interactions occur between genes encoding proteins involved in a common complex process (17, 18). The *scr2* mutant strain that contains chromosomal deletions of both *SCR1* and *SCR2* and carries the *scr2*-II.13 allele on a replicative plasmid is viable at 32 °C, having a generation time doubled at this temperature (1). Clones unable to grow at 32 °C were selected after UV mutagenesis of the *scr2* mutant strain. To distinguish between mutants with a tight temperature-sensitive lethal growth phenotype and synthetic lethal ones, the *scr2*-II.13 allele was replaced by the wild-type allele by plasmid shuffling (selection for Leu+ Ura− transformants), and the growth phenotype produced by the second mutation alone was studied. The *sls1*-1 mutation (for synthetic lethal with the 7S RNA mutation) was shown to confer an extreme Ts growth phenotype only in association with the *scr2*-II.13 mutation (Fig. 1, *1 versus 2*). However, the *sls1* single mutant still displayed a temperature-sensitive growth, having its generation time doubled at 28 °C in a *SCR2* context as compared with a wild-type strain (Fig. 1, *2 versus 3*).

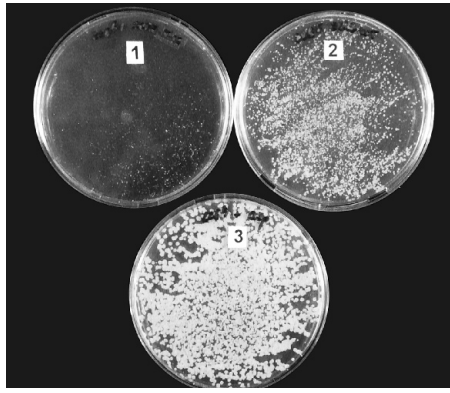*Isolation of the SLS1 Gene*—The *sls1*-1 mutation was shown

FIG. 1. **Colonies formed on rich medium after incubation at 28 °C by the double mutant *sls1*-1, *src2*-II.13 (*1*), the *sls1*-1 mutant (*2*), and the wild-type strain (*3*).**
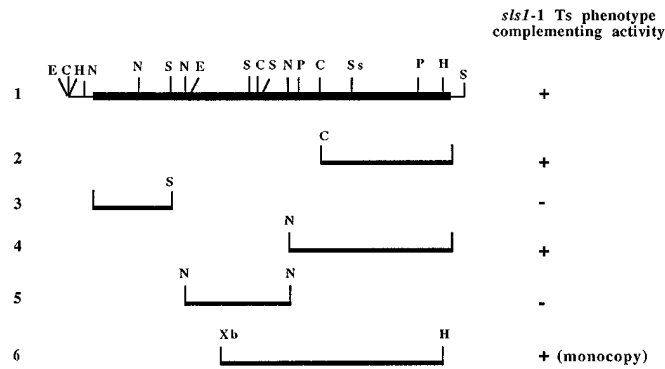

FIG. 2. **Map of the chromosomal insert cloned in the plasmid complementing the *sls1* mutant temperature sensitivity (*1*) and subfragments tested for complementation of the *sls1*-1 growth defect in multicopy (*2–5*) or monocopy (*6*).** *C*, *Cla*I; *E*, *Eco*RI; *H*, *Hin*dIII; *N*, *Nhe*I; *P*, *Pvu*II; *S*, *Sal*I; *Ss*, *Ssp*I; *Xb*, *Xba*I.

FIG. 3. *A*, detailed restriction map of the sequenced minimal complementing region. The *SLS1* open reading frame is represented by a *large dark arrow*. The upstream and downstream regions are visualized by a *thin box*. The *Cla*I-*Hin*dIII fragment containing a internal deletion and the *URA3* gene was used to inactivate the chromosomic copy of *SLS1*. *B*, nucleotide sequence of the *SLS1* gene. Coordinate +1 corresponds to the translation initiation codon. The potential TATA box is *circled*, and the potential polyadenylation site is *underlined*. The predicted amino acid sequence is shown in single-letter code. The putative signal peptide and ER retention motif are *boxed*.

to be recessive and monogenic by genetic analysis (not shown). The *sls1* mutant was thus transformed with a *LEU2*-based replicative *Y. lipolytica* genomic library. 25,000 Leu+ transformants were selected at 28 °C on leucine-free medium and were tested for growth at 32 °C by replica plating on rich medium. Four independent clones were able to grow at this temperature. Temperature-resistance depended on the presence of the recombinant plasmids because Leu− segregants recovered the initial Ts growth phenotype. The four plasmids isolated from these colonies contained the same 5-kb genomic insert as shown by restriction enzyme analysis. To localize the *sls1*-1 complementing gene in this insert, several overlapping fragments were subcloned and recombinant plasmids were tested for complementation of the *sls1* temperature sensitivity. A 1.9-kb fragment was sufficient to confer temperature resistance to the *sls1* mutant (Fig. 2). Integration of the 3.4-kb *Xba*I-*Hin*dIII fragment at the chromosomal *LEU2* locus restored a wild-type growth at 32 °C. This insert was therefore able to complement the *sls1*-1 mutation as a single copy.

Primary Structure of the SLS1 Gene Product—The 1967-base pair insert was sequenced and shown to contain a single complete open reading frame of 1278 bp (Fig. 3*A*), potentially encoding a 426-amino acid polypeptide. A putative TATA box was found 135 bp upstream of the start codon, and a putative transcription termination element was present 14–58 bp downstream of the TAA translation stop codon (Fig. 3*B*). The deduced amino acid sequence shared no significant homology with proteins present in the data base. However, the amino-terminal sequence of the *SLS1* protein showed the features of a 17-amino acid signal peptide, containing a stretch of 8 hydro-
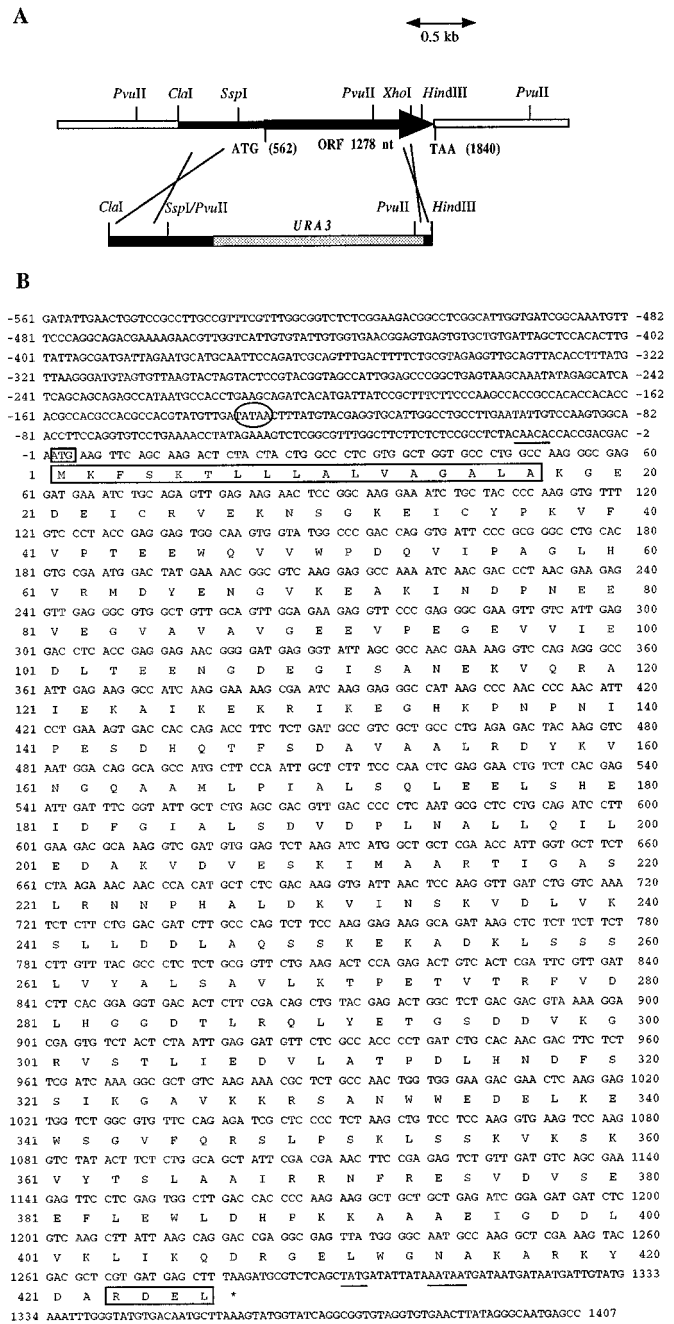
phobic residues (19). Such an amino-terminal extension characterizes secreted proteins. A tetrapeptide motif RDEL was observed at the carboxyl terminus of the predicted sequence. A related sequence, KDEL, has been shown to be implicated in soluble protein retention in the endoplasmic reticulum (20). Because no other hydrophobic region is present, Sls1p is likely to be an ER lumenal protein. No potential *N*-linked glycosylation site was found.

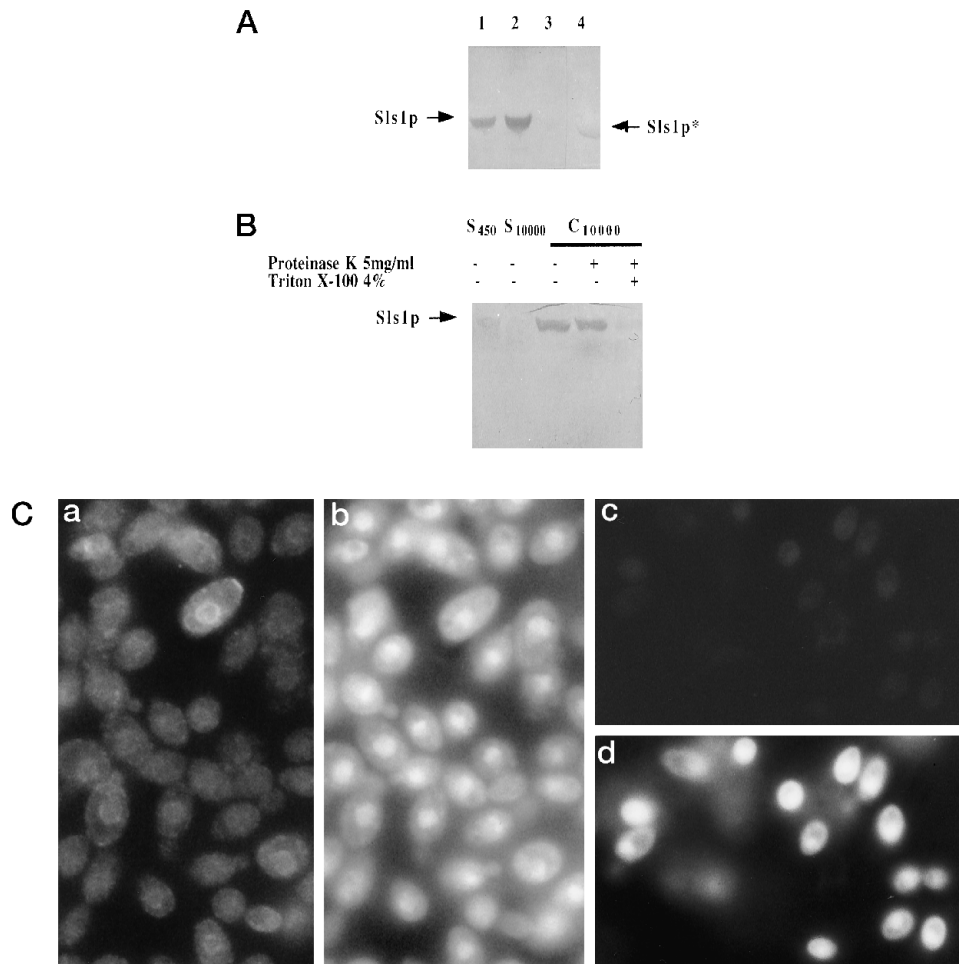*Localization of the SLS1 Gene Product*—To verify the pre-

FIG. 4. **Localization of the Sls1 protein.** *A*, characterization of the anti-Sls1p antibodies produced against a glutathione *S*-transferase-Sls1p fusion protein. Western blot analysis of whole cell extracts from various *Y. lipolytica* transformants containing different *SLS1* mutant constructs. The strain in *lane 1* contains the wild-type *SLS1* allele on the chromosome, the strain in *lane 2* also contains the *SLS1* gene on a 3–4-copy plasmid, the strain in *lane 3* contains the deleted Δ*sls1* allele on the chromosome, and the strain in *lane 4* contains a 3′-truncated chromosomal copy of *SLS1*. Equal amounts of total protein were applied on SDS-PAGE. *B*, cell fractionation and protease protection of Sls1p. A whole cell extract from the wild-type strain 136463 was made by a gentle method and cleared from unbroken cells and cell wall fragments by centrifugation at 450 × *g*. Supernatant (S_{450}) was recollected and subfractionated by a 20-min centrifugation at 10,000 × *g* leading to two fractions (S_{10000} and C_{10000}). Samples of the resuspended latter fraction were treated on ice for 1 h with 0.5 mg/ml of proteinase K in the absence or in the presence of 4% Triton X-100. Samples were analyzed by SDS-PAGE followed by Western blotting with Sls1p antiserum. *C*, localization of Sls1p by immunofluorescence in cells expressing the chromosomic *SLS1* gene. Cells were grown overnight in rich medium, prepared for immunofluorescence, and treated with rabbit anti-Sls1p antibodies, followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG to localize the Sls1 protein (*a*) and 4,6-diamidino-2-phenylindole to visualize nuclear DNA (*b* and *d*). The Sls1p signal was abolished by exclusion of the primary antibodies or by staining of the Δ*sls1* strain (*c*).

dictions concerning the intracellular localization of Sls1p, antibodies were raised against a large region of the Sls1 protein extending from residue 21 to residue 403, fused to the *S. japonicum* glutathione *S*-transferase protein. The serum reacted with a unique protein of approximately 55 kDa in whole extracts of wild-type cells (Fig. 4*A*, *lane 1*). This signal was amplified in cells carrying the *SLS1* gene on a centromeric plasmid (Fig. 4*A*, *lane 2*). In cells expressing a carboxyl-terminal deletion of Sls1p lacking the last 23 amino acid residues, the signal appeared at a lower molecular weight (Fig. 4*A*, *lane 4*). The amount of the truncated protein was reduced compared with the wild-type protein level. In a cell fractionation and protease sensitivity experiment, Sls1p was shown to reside within a membranous cellular compartment. Sls1p was sedimented after a low speed centrifugation (10,000 × *g*) and remained resistant to protease degradation in the absence of detergent (Fig. 4*B*). However, after membranes solubilization by Triton X-100, Sls1p became accessible to proteinase K. Immunofluorescence microscopy was performed with anti-Sls1p antibodies to determine the localization of the *SLS1* gene prod-

uct. Wild-type cells showed a specific Sls1p perinuclear staining (Fig. 4*C*). Because the ER is an extension of the nuclear envelope, this staining pattern is consistent with a localization of the protein into the ER. A similar pattern was obtained in *S. cerevisiae* for the soluble ER resident protein Kar2p (21) or for the ER membrane protein Sss1p (22). In cells expressing the 3′-truncated copy of the *SLS1* gene, Sls1p immunoreactivity was more diffuse (not shown).

Levels of Sls1p Expression—ER lumenal proteins such as the chaperone BiP or the protein disulfide isomerase are involved in folding of translocated polypeptides and are induced under conditions leading to protein misfolding. In the yeast *Schizosaccharomyces pombe* or in *S. cerevisiae*, increased levels of the BiP/Kar2 protein result from an induction of its transcripts (21, 23). To test such a regulation for the *SLS1* gene, intracellular levels of Sls1p were first examined by immunoblotting under conditions of heat shock and inhibition of glycosylation. A 2-fold increase of Sls1p intracellular amounts was observed 1 h after incubation at 34 °C compared with levels in cells incubated at 28 °C (Fig. 5*A*, *lanes 2*, *4*, and *6 versus lanes 1*, *3*, and *5*) and

A

Time of incubation (min) 90 75 60
Temperature 34 C 28 C 34 C 28 C 34 C 28 C
6 5 4 3 2 1

Sls1p →

B

Tunicamycin (10 μg/ml) - +
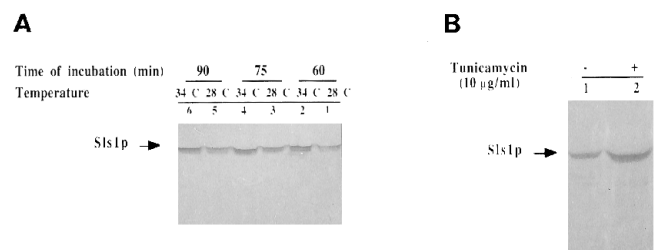1 2

Sls1p →

FIG. 5. **Levels of intracellular Sls1p after heat shock and tuni-camycin treatment.** *A,* half of an overnight culture in rich medium was incubated at 34 °C (*lanes 2, 4,* and *6*), whereas the second half remained at 28 °C (*lanes 1, 3,* and *5*). Samples were taken every 15 min for 90 min. *B,* half of an overnight culture in rich medium was incubated for 3 h at 23 °C in the presence of tunicamycin (10 μg/ml) (*lane 2*), whereas the second half was left untreated (*lane 1*). Whole cell extracts were made and subjected to SDS-PAGE (8% polyacrylamide gel). Proteins were transferred to mitrocellulose and probed with rabbit anti-Sls1p antibodies and goat anti-rabbit IgG conjugated with peroxidase. In these experiments, equal amounts of total protein were loaded for each extract.

was maintained for at least 30 min. Half of an overnight wild-type cell culture, freshly diluted, was treated with tunicamycin for 3 h at 23 °C. Levels of Sls1p detected by Western blot in cell extracts from this culture were 2–3-fold higher than amounts revealed in cells of the untreated second culture (Fig. 5*B, lane 2 versus lane 1*). Similar results were obtained when *SLS1* induction was monitored using a *SLS1::lacZ* transcription fusion (not shown).

Sls1p Is Required for Growth at Elevated Temperatures—Because a short Sls1 protein (of approximately 20 kDa) is still present in the *sls1*-1 mutant, a *sls1* null mutant was constructed to determine if the protein was required for cell viability. A 2.45-kb *Cla*I-*Hin*dIII fragment carrying a deletion of the first 289 amino acids and the *URA3* gene (Fig. 3*A*) was used to transform a haploid strain carrying a *SLS1* wild-type copy on a *LEU2*-based replicative plasmid. Ura+ transformants were selected. To eliminate clones that would have integrated the deleted fragment in the resident *LEU2*-plasmid, stable Ura+ clones were retained after culture in rich medium and replica plating on 5-fluoroorotic acid medium in the presence of leucine. Loss of the *LEU2, SLS1* plasmid was then selected after incubation at 25 °C. One of the Ura+ Leu− segregants showed a temperature-sensitive growth phenotype. Integration of the deleted copy in place of the wild-type one was confirmed by Southern blot analysis. Immunoblot of extracts from Δ*sls1* cells with anti-Sls1p antibodies revealed no signal (Fig. 4*A, lane 3*). The function of the Sls1p is therefore not required for cell viability but is essential for optimal growth at elevated temperatures.

Secretory Protein Synthesis Is Inhibited in Δsls1 Cells—Because the *scr2*-II.13 mutation caused a specific inhibition of the synthesis of a cotranslationally translocated secretory protein (1) and because the *sls1*-1 mutation conferred a synthetic growth defect with the 7S RNA mutation, we looked for similar defects in the *sls1* null mutant. Δ*sls1* cells were compared with *SLS1* cells for synthesis, maturation, and secretion rates of the AEP by a pulse-chase labeling and immunoprecipitation experiment after a 1 h shift to 28 °C. AEP is synthesized as a 53-kDa preproprotein with a signal sequence and a pro-domain upstream from the mature domain. In wild-type cells, the earliest precursor immunoprecipitated (pAEP, 55 kDa) lacks the signal sequence and is core-glycosylated, as expected for a cotranslationally translocated protein (16, 24). Subsequent cleavage by a Xpr2-like Golgi endoprotease results in a 20-kDa propeptide and a 32-kDa mature form, both of which are secreted.

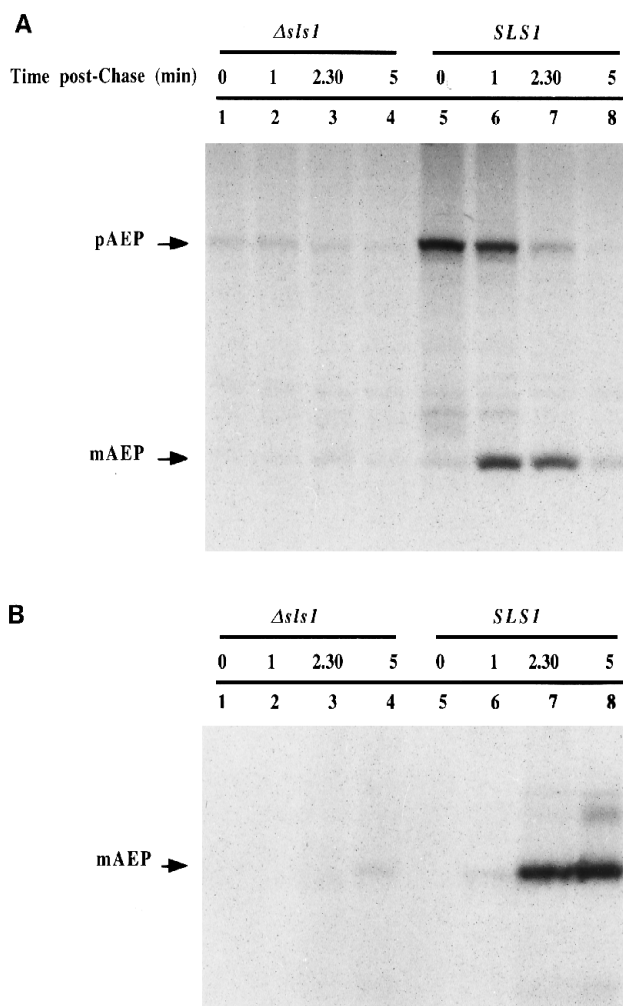Δ*sls1* and *SLS1* cells were labeled in inducing medium for 45

A

Δ*sls1* *SLS1*
Time post-Chase (min) 0 1 2.30 5 0 1 2.30 5
1 2 3 4 5 6 7 8

pAEP →

mAEP →

B

Δ*sls1* *SLS1*
0 1 2.30 5 0 1 2.30 5
1 2 3 4 5 6 7 8

mAEP →

FIG. 6. **Pulse-chase labeling and immunoprecipitation analysis of AEP synthesis and secretion in *SLS1* and Δ*sls1* strains.** *Y. lipolytica SLS1* cells (*lanes 5–8*) and Δ*sls1* cells (*lanes 1–4*) were grown overnight at 18 °C in inducible medium, concentrated in fresh medium, and transferred for 1 h at 28 °C before a 45-s labeling pulse with L-[4,5-³H]leucine. Cells were chased by the addition of a 300-fold excess of cold leucine, and samples were taken 0, 1, 2.30, and 5 min after the chase. *A,* intracellular extracts were immunoprecipitated with polyclonal anti-AEP antibodies and protein A-Sepharose. *B,* supernatant proteins were trichloroacetic acid-precipitated. Precipitates were resolved by SDS-PAGE and visualized after fluorography at −80 °C. Positions of the AEP 55-kDa precursor and the 32-kDa mature form are indicated by *arrows.*

s and chased. Fig. 6*A* shows a 7-fold reduction of the amount of labeled intracellular forms in Δ*sls1* cells compared with *SLS1* cells (*lanes 1–4 versus lanes 5–8*). The level of the secreted 32-kDa mature form in the growth medium of Δ*sls1* cells, detected after trichloroacetic acid precipitation, was also lower than these revealed in supernatant of *SLS1* cells (Fig. 6*B, lanes 1–4 versus lanes 5–8*). Total protein synthesis was similar in the two strains. Because AEP precursors in Δ*sls1* cells could bind to concanavalin A-Sepharose (not shown), they corresponded to translocated forms. Maximal level of both precursor and mature forms was only obtained at 2.30 min in Δ*sls1* cells (Fig. 6*A, lane 3*) compared with 1 min in *SLS1* cells (Fig. 6*A, lane 6*). Therefore, detection of total newly synthesized AEP precursors was delayed in the absence of the *SLS1* gene product. This observation suggests that translocation is affected in a Δ*sls1* context. In addition, a delay in AEP processing was observed. In contrast to *SLS1* cells, where the mature form was predominant at 1 min in cell extracts (Fig. 6*A, lane 6*) and secreted at 2.30 min in the medium (Fig. 6*B, lane 7*), most of
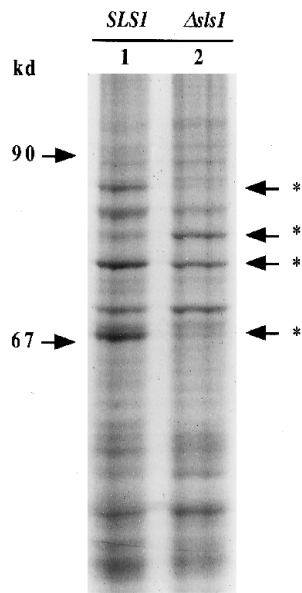
FIG. 7. **Pattern of glycosylated proteins in *SLS1* cells (*lane 1*) and in Δ*sls1* cells (*lane 2*) at 26 °C.** [35S]Methionine-labeled total proteins from each strain were incubated with concanavalin A-Sepharose beads. Bound proteins were eluted by heating, applied to a 6% SDS-polyacrylamide gel, and visualized after fluorography.
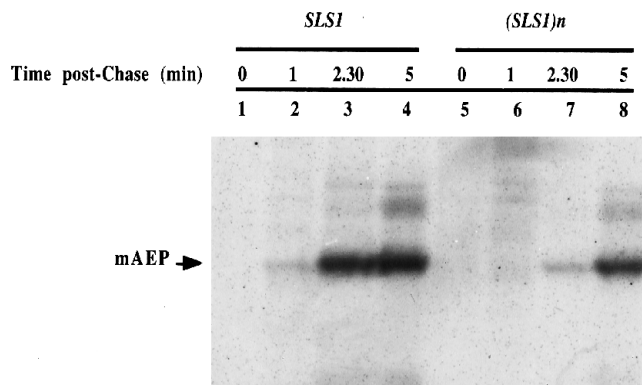

FIG. 8. **AEP secretion in Sls1p overexpressing cells.** *SLS1* monocopy cells (*lanes 1–4*) and *SLS1* multicopy cells (*lanes 5–8*) were pulse-labeled for 45 s and chased as previously described. Samples were taken at 0, 1, 2.30, and 5 min post chase and centrifuged. Proteins from each supernatant were trichloroacetic acid-precipitated and subjected to SDS-PAGE and fluorography. Mature AEP is signaled by an *arrow*.

AEP mature form was immunoprecipitated at 2.30 min in Δ*sls1* cell extracts (Fig. 6*A*, *lane 3*) and appeared at 5 min in supernatant (Fig. 6*B*, *lane 4*). This delay in maturation reflects an increased transit time upstream from the Golgi.

The effect of the deletion of the *SLS1* gene on other secretory proteins was tested by looking at the amount of glycosylated [35S]methionine-labeled proteins bound to concanavalin A-Sepharose in Δ*sls1* cells compared with *SLS1* cells. The level of several major glycoproteins appeared to be largely reduced in the absence of the *SLS1* gene product (Fig. 7, *lane 2 compared with lane 1*), suggesting that synthesis of these preproteins was impaired. In contrast, the amount of at least one glycoprotein was increased in Δ*sls1* cells.

Secretion of AEP Is Delayed in SLS1 Overexpressing Cells— AEP synthesis and secretion were studied in the presence of a 3-fold higher level of the Sls1 protein, as estimated by immunoblotting (Fig. 4*A*), in order to detect any modification of the secretion process. Pulse-chase labeling experiments were performed under the same conditions as for the *sls1* null mutant. The ratio of AEP incorporation, measured after immunopre-
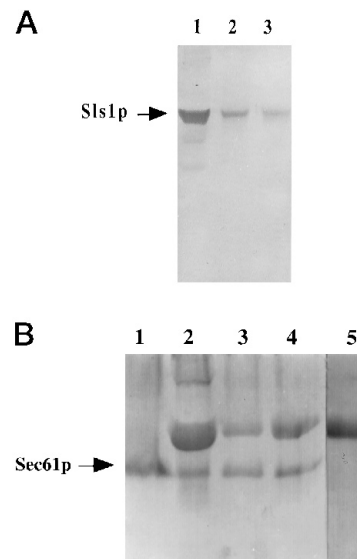

FIG. 9. **Sls1p and Sec61p co-precipitation.** *A*, a membrane-rich fraction was prepared from wild-type cells. Samples were subjected or not to cross-linking by the cleavable reagent dithiobis(succinimidyl propionate) (0.2 mg/ml) simultaneously to solubilization in 1% Triton X-100 and were immunoprecipitated by anti-*S. cerevisiae* Sec61p antibodies. Precipitates were resolved on SDS-PAGE, transferred to nitrocellulose, and blotted with anti-Sls1p antibodies. *Lane 1*, crude extracts; *lane 2*, Sec61p immunoprecipitates after cross-linking; *lane 3*, Sec61p immunoprecipitates without dithiobis(succinimidyl propionate) treatment. *B*, Sec61p blotting with antibodies raised against a 21-amino acid NH2-terminal peptide[2] on crude extracts (*lane 1*), on extracts solubilized with 1% Triton X-100 and immunoprecipitated either by anti-*Y. lipolytica* Sec61p antibodies (*lane 2*), by anti-*S. cerevisiae* Sec61p antibodies (*lane 3*), or by anti-Sls1p antibodies (*lane 4*). In *lane 5*, immunoprecipitates analyzed in *lane 2* were probed with anti-*Y. lipolytica* Sec61p preimmune serum.

cipitation, to total protein labeling was similar in both extracts of overexpressing cells and wild-type cells, suggesting that the Sls1 protein is not limiting in the cotranslational translocation process. However, in these cells, intracellular maturation of the 55-kDa precursor form by the endoprotease and subsequent secretion of the 32-kDa mature form in the growth medium were delayed compared with *SLS1* cells (Fig. 8, *lanes 5–8 versus lanes 1–4*).

*Sls1p Is Localized Close to the Major Component of the Translocation Pore—*In order to test the proximity of the Sls1 protein to the translocation apparatus as expected for a protein involved in translocation, co-precipitation of Sls1p with the major component of the translocation channel Sec61p (25) was performed. A Sec61p homologue has been recently identified in *Y. lipolytica*.[2] The *Yl* Sec61p amino acid sequence shares 22 amino acid residues out of 28, between residues 5 and 32, with the *Sc* Sec61p NH2-terminal region recognized by the antibodies. A membrane-rich fraction was made from wild-type cells and subjected or not to cross-linking by the cleavable reagent dithiobis(succinimidyl propionate) simultaneously to solubilization with 1% Triton X-100. Extracts were then immunoprecipitated with an excess of anti-*Sc* Sec61 antibodies. Immunoprecipitates, resolved on SDS-PAGE and transferred to nitrocellulose, were blotted with anti-Sls1p antibodies. The Sls1 protein was co-precipitated with the Sec61p homolog of *Y. lipolytica* as shown in Fig. 9*A*. Because a signal corresponding to Sls1p (*lane 1*) was obtained either with or without prior cross-linking (*lanes 2* and *3*), the Sls1 protein appeared to be tightly associated with the Sec61 protein. The reverse experiment was done with antibodies raised against a NH2-terminal

[2] D. Swennen, manuscript in preparation.

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Y. lipolytica    K A I K E K R I K E G H K P N P N I P E S D H Q T F S D A V A A L R D Y - K V N G Q A A
S. cerevisiae    I L K I Y S D E A T G L R V D P L V V L F L A V G I F S V V A L H V T S K V A G K L F
A. thaliana      M L Q F Y T D D A P G L K I S P N V V L I M S I G F I A F V A V L H V M G K L Y F V K
Zea mais           L Q F Y T D E T A G R K M S P N T V L I I S I x F A
Sea Urchin               D S P G L K V G P V P V L V M C L I F I A S V F M L H I W G K
Dog              M W R F Y T E D S P G L K V G P V P V L V M S L L F I A S V F M L H I W G K Y T R S
Human T78854         D S P P G L K V A P C P x L M K S S C S I A P V F M L H I W G K Y T R S
Human T62690             L K V G F V A V L L L E V L C E A S V F L M R L Y
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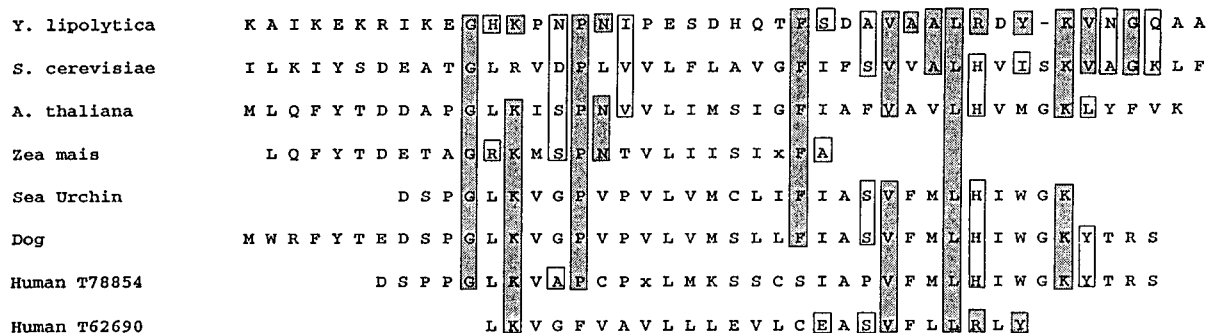
FIG. 10. **Sequence alignment of a Sls1p domain with a region of Sec61β proteins from various organisms and human cDNA sequences.** Identical amino acids are *shaded* in *dark gray* and similar residues in *light gray.*

peptide of *Y. lipolytica* Sec61p. These antibodies revealed a same product either in crude extracts (Fig. 9*B, lane 1*) or in *Yl* Sec61p (*lane 2*), *Sc* Sec61p (*lane 3*), or Sls1p immunoprecipitates (*lane 4*). No signal appears in *Y. lipolytica* Sec61p immunoprecipitates when blotting was done with preimmune serum (*lane 5*).

DISCUSSION

Genetic data indicate that major secretory proteins are targeted and translocated across the ER membrane in a SRP-dependent way in the yeast *Y. lipolytica* (12). This yeast therefore represents a good model for a genetic approach of cotranslational translocation molecular mechanisms. A SRP-deficient strain carrying the mutant *scr2*-II.13 allele on a replicative plasmid (1) was used to look for secondary mutations that specifically exacerbate the Ts growth phenotype displayed by this first mutation. Such genetic interactions leading to synthetic enhancement have been observed *a posteriori* for *SEC17* and *SEC18*, whose products are both involved in transport vesicles fusion (17), or between two specific alleles of the *SEC63* and *KAR2* genes (26). In our case, secondary mutations could affect products that either act at the same targeting step or function in a different but coordinated step. Several mutations were obtained that conferred high sensitivity to elevated temperature only in combination with the 7S RNA mutation (*sls*); one called *sls1*-1 was studied in more detail.

The *SLS1* gene encoded a 426-amino acid residue polypeptide with a functional signal sequence at its NH$_2$ terminus and a functional ER retention signal at its COOH terminus as evidenced by immunofluorescence microscopy. Like lumenal ER proteins in other yeasts, the *Y. lipolytica* Sls1p hydrophilic protein is probably localized into the ER through a retention mechanism involving a specific receptor (27). This receptor should recognize the RDEL motif and allow retrieval of the protein from a late compartment. Indeed, removal of this ER retention signal results in decreased amounts of intracellular Sls1p. The truncated protein probably continues on the secretory pathway transiting through the Golgi and secretion vesicles. However, no significant levels of Sls1p were detected in the growth medium, probably due to instability of the protein outside in the medium or degradation along the secretory pathway. The strain expressing this truncated Sls1 protein did not show any growth defect, suggesting that the secreted form of Sls1p performed its function during its transit through the ER.

The *sls1* null mutation conferred a temperature-sensitive growth phenotype similar to that of the *sls1*-1 mutant, indicating that the 20-kDa *SLS1* product detected in the latter cells was not functional. The temperature sensitivity of Δ*sls1* cells may reflect an improving role for Sls1p in an essential cellular process, such as preprotein translocation, that could become limiting at 30 °C in the absence of Sls1p. Alternatively, Sls1p

may perform an essential function only at elevated temperatures in a heat shock pathway. In a Δ*sls1* context, synthesis of several preproteins was severely reduced as in the *scr2* mutant. The main conclusion from this observation is that the Sls1 protein is likely to be involved in the cotranslational translocation process, accounting for the genetic interaction between the two mutations. The translation defect of the *scr2*-II.13 mutant could be explained by a decrease in initiation of preprotein translocation. To explain the translation defect in the absence of Sls1p, we favor the hypothesis that Sls1p helps translocating polypeptides on the lumenal side of the ER membrane, completing their translocation. Consistent with this scheme, a delay is observed for the appearance of total newly synthesized precursors in the absence of the *SLS1* gene product. The function of mammalian ER lumenal proteins in cotranslational translocation, mediating the net transfer of the polypeptide into the ER lumen, has already been proposed (28). Sls1p might thus increase the translocation initiation rate on the opposite side of the ER membrane by freeing the translocon from the ER side. Co-precipitation of Sls1p with the major component of the translocation apparatus, which reveals that Sls1p resides in the vicinity of the translocation site, fits well with a function of the protein in preprotein translocation. Interestingly, Sls1p displays a domain similar to the COOH-terminal moiety of the family of Sec61β polypeptides (Fig. 10). Sec61β was described as a companion of Sec61p (Sec61α) in the translocon (29). We propose that this domain is involved in Sec61p binding for Sls1p. This hypothesis will be tested in the near future.

Our results are compatible with a second function of Sls1p in protein folding after translocation has been completed, its defect accounting for the delay observed in the processing of the 55-kDa precursor form in Δ*sls1* cells. Additional evidence for a role of Sls1p during precursor transit through the ER was provided by the delay of precursor processing into the mature form in cells overexpressing Sls1p. Prevention of secretion of some secretory proteins has been described in mammalian cells overexpressing the BiP protein (30) and was supposed to result from the stabilization of the complex between newly translocated preprotein and BiP. Induction of Sls1p levels under conditions leading to ER accumulation of misfolded preproteins was also consistent with such a property.

Our results show that synthetic lethality may be used as a screen for isolation of new mutations and that such genetic interaction could be observed for two products that are physically separated but involved in the same coordinated pathway.

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