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Interaction of Kar2p and Sls1p Is Required for Efficient Co-translational Translocation of Secreted Proteins in the Yeast *Yarrowia lipolytica**

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The yeast *Yarrowia lipolytica* is a model organism for *in vivo* study of the signal recognition particle-dependent targeting pathway. In this report, we defined solubilization conditions and set up a fractionation procedure of *Y. lipolytica* microsomes to determine the amounts of Sec61p-containing translocation pores linked to ribosomes. In contrast to *Saccharomyces cerevisiae*, from 70 to 80% of Sec61p associates with ribosomes in this yeast. The chaperone protein Kar2p and the Sls1p product, a resident protein of the endoplasmic reticulum lumen, partially fractionate with this Sec61p population. Moreover, Sls1p can be co-immunoprecipitated with Kar2p, and the two polypeptides are shown to directly interact in the yeast two-hybrid system. A site-directed mutagenesis was performed on the *SLS1* coding sequence that allowed us to define a functional domain in Sls1p. Indeed, co-translational translocation of a reporter protein is affected when one of these mutant proteins is expressed. Moreover, this protein has lost its capacity to interact with Kar2p, and the two lumenal polypeptides might thus cooperate to promote secretory protein co-translational translocation.

To initiate their pathway, secretory proteins are first targeted to the endoplasmic reticulum (ER)[1] membrane in eukaryotic organisms. In mammalian cells, a cytoplasmic particle (the Signal Recognition Particle or SRP) first recognizes these proteins when the signal peptide emerges from the ribosome and then ensures their delivery to the ER membrane through its interaction with the SRP receptor (1). SRP binding to the nascent chain-ribosome complex causes a translational pause that is released upon docking of SRP (2). As translation resumes, the complex is transferred to the translocation site where crossing through the ER membrane takes place. Sec61α, one of the three polypeptides of the translocation pore, is a polytopic membrane protein; the two others, Sec61β and γ, span the ER membrane once. Three to four units of this heterooligomer are needed to form the aqueous pore (3). *In vitro* reconstitution experiments, using purified mammalian components, show that the SRP receptor and the Sec61 complex are sufficient to achieve translocation of some preproteins, while the TRAM protein is required for translocation of other preproteins (4). *In vivo*, many other soluble or membrane proteins could be involved in this process to adjust the translocation rate to cell growth.

In the model yeast, *Saccharomyces cerevisiae*, components involved in SRP-dependent targeting are not essential, and several secretory proteins were shown to cross the ER membrane post-translationally (5, 6). This translocation mode relies both on cytosolic chaperones whose binding delays preproteins folding (7) and on membrane proteins that ensure specific insertion of secretory proteins at the translocation site. A heptameric complex containing the trimeric Sec61 complex and four other polypeptides, Sec62p, Sec63p, Sec71p, and Sec72p, allows *in vitro* post-translational translocation of several preproteins in the presence of the lumenal chaperone Kar2p and ATP (8). All these polypeptides were previously identified in genetic screens and through biochemical approaches (9, 10). A contribution of proteins of the Hsp70 family has been described for two preprotein transport machineries. The Kar2p and mitochondrial matrix mHsp70 proteins, respectively, promote translocation across the endoplasmic reticulum membrane and across the mitochondrial inner membrane in yeast. While the transmembrane protein Sec63p was identified as the Kar2p-binding partner in the ER membrane (11), two components of the Tim complex, Tim44 and Tim17, were found to function as membrane anchor for mHsp70 (12, 13).

In the yeast *Yarrowia lipolytica*, inactivation of the two genes encoding the 7 S RNA component of the SRP is lethal (14), whereas deletion of the *SRP54* and *SEC65* genes result in very low growth (15). SRP is also essential in the yeast *Schizosaccharomyces pombe* (16). Study of *Y. lipolytica* conditional lethal 7 S RNA mutants provided the first *in vivo* evidence of SRP involvement in the targeting step of the co-translational translocation process (17). Pursuing this genetic approach, secondary mutations that led to synthetic lethality in combination with the 7 S RNA mutation were selected. This screen allowed cloning of the *SLS1* gene whose product, located in the lumen of the endoplasmic reticulum, was shown to participate in preprotein translocation (18). To gain insights in Sls1p function, putative associations with known ER resident proteins were tested. In the present paper, we show that Sls1p directly interacts with the chaperone protein Kar2p. We also show by a fractionation procedure that the majority of translocation pores in *Y. lipolytica* are linked to ribosomes and that Sls1p and Kar2p partially co-fractionate with ribosome-bound Sec61p. To investigate the structure/function relationship, we constructed different Sls1p mutant proteins and compared their ability to interact with Kar2p with their capacity to promote

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[1] The abbreviations used are: ER, endoplasmic reticulum; SRP, signal recognition particle; RAMP, ribosome-associated membrane protein; AEP, alkaline extracellular protease; PAGE, polyacrylamide gel electrophoresis.

secretory protein translocation. One of these mutant proteins, Sls1−5p, that does not further associate with Kar2p confers a decrease in the synthesis of a secreted protein, suggesting that such an interaction is required for efficient co-translational translocation.

MATERIALS AND METHODS

Strains and Plasmids—Plasmid pINA1077 contains a 2.2-kb *Cla*I-*Sal*I fragment comprising the entire *SLS1* open reading frame inserted into the multicloning site of the phagemid Bluescript SK$^-$ (Stratagene) (18). Mutant forms of this plasmid were obtained by *in vitro* site-directed mutagenesis using the Kunkel method (19). Five oligonucleotides were used as primers to introduce mutations or deletions (see Table I), and the presence of the mutations was confirmed by sequencing. The *Cla*I-*Sal*I fragments, containing the deletions, were subcloned in a *Y. lipolytica* integrative plasmid and integrated in the genome of the Δ*sls1* strain previously constructed: *MatB*, *scr1::ADE1*, *SCR2*, *his*-1, *leu2*, *ura3*, *sls1::URA3* (18).

Plasmid pAS2ΔΔ (*TRP1*, Amp^r) was used for expression of GAL4-DNA binding domain fusion proteins and plasmid pACT2 (*LEU2*, Amp^r) for expression of GAL4-activating domain fusion proteins. The *S. cerevisiae* strain PJ69−4A (*MAT*a, *trp1*−901, *leu2*−3, 112, *ura3*−52, *his3*−200, *gal4*Δ, *gal80*Δ, *LYS2::GAL1-HIS3*, *GAL2-ADE2*, *met2::GAL7-lacZ*) was used for the two-hybrid analysis (20). Two couples of primers were designed to amplify the *KAR2* nucleotide sequence between codon 27 and codon 656 (Kar2p-1: 5′-CCG G**CC ATG G**GC GTT CAG GCT GAT GAC GTG, and Kar2p−4: 5′-CG**G GAT CC**C ACC GTC GTT GGA CTC GTC TC) and the *SLS1* coding sequence from codon 19 to codon 411 (Sls1-α: 5′-CCG G**CC ATG G**GC GAG GAT GAA ATC TGC AGA and Sls1-β: 5′-CG**G GAT CC**A TAA CTC GCC TCG GTC CTG). After amplification, polymerase chain reaction products were digested by *Nco*I and *Bam*HI to allow in-frame cloning in the two-hybrid vectors multicloning site. Empty and recombinant two-hybrid vectors were co-transformed into yeast cells by the lithium acetate method, and transformants were selected on minimal medium supplemented with histidine, methionine, uracil, and adenine for selection of the plasmids and on minimal medium supplemented with histidine, methionine, and uracil, or on minimal medium supplemented with adenine, methionine, and uracil plus 5 mM 3-aminotriazole for direct selection of the interaction (20).

Antibodies—The 17 C-terminal amino acid residues from YlSec62p were replaced by a peptide corresponding to the 13 C-terminal amino acid residues of ScSec62p, allowing detection of the *Y. lipolytica* Sec62p using anti-*S. cerevisiae* Sec62p antibodies. ScSec62p and YlKar2p antibodies were kind gifts of R. Schekman and of D. Ogrydziak, respectively. Anti-YlSec61p were raised against the 14 N-terminal amino acid residues and were purified on protein A-Sepharose as described in Görlich and Rapoport (4).

Preparation and Differential Extraction of Microsomes from Yarrowia lipolytica—Yeast cells grown in rich medium and harvested during exponential phase were lysed by a 10-min vortexing with glass beads in homogenizing buffer (250 mM Hepes-KOH, pH 7.5, 25 mM potassium acetate, 5 mM magnesium acetate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 50% glycerol). Supernatant from a low speed centrifugation was submitted to a 30-min centrifugation at 15,000 rpm. The resulting microsomal pellet was resuspended in homogenizing buffer at a final concentration of 1–2 equivalents per microliter (1 equivalent is defined as 1 microliter of a membrane suspension with an absorbance at 280 nanometers of 50). 100 equivalents were then diluted in 500 microliters of 2× solubilizing buffer (25 mM Hepes-KOH, pH 7.5, 400 mM potassium acetate, 8 mM magnesium acetate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol), detergents were added, and samples were incubated for 30 min on ice. A 40-min centrifugation at 70,000 rpm in a 100.4 rotor in a Beckman tabletop ultracentrifuge then yielded a supernatant, containing solubilized material, and a ribosomal pellet. After resuspension in a high salt buffer containing 2 mM puromycin and 0.2 mM GTP, the pellet was incubated for 30 min on ice and then for 30 min at 28 °C. Ribosome-associated membrane proteins (RAMPs) were detected in the supernatant of a 1-h centrifugation at 100,000 rpm in a 100.4 rotor in a Beckman tabletop ultracentrifuge.

Binding to Concanavalin A-Sepharose, Immunoprecipitation, and Western Blotting—Supernatant fractions and pellet samples were diluted in 500 microliters of 20 mM Hepes-KOH, pH 7.5, 150 mM potassium acetate, 5 mM magnesium acetate, 1 mM phenylmethylsulfonyl fluoride buffer before addition either of 50 microliters of concanavalin A-Sepharose or 100 microliters of anti-YlSec61p antibodies and overnight incubation at 4 °C. For Kar2p immunoprecipitation, microsomes in solubilizing buffer were incubated for 30 min on ice in the presence of 2% Triton X-100. Unsolubilized material was discarded by a 30-min centrifugation at 15,000 rpm in a 100.4 rotor in a Beckman tabletop ultracentrifuge, and anti-Kar2p antibodies plus protein A-Sepharose were added to the clear supernatant after a 4-fold dilution in cold phosphate-buffered saline. Immunoprecipitation was performed overnight at 4 °C. Sepharose beads were washed three times with 500 microliters of phosphate-buffered saline, and precipitates were eluted in sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 3% SDS, Bromphenol blue 0.02%) for 20 min at 55 °C. Samples were then applied on a 10% polyacrylamide denaturant gel, and proteins were transferred onto nitrocellulose membrane after the migration. Sec61p, Sec62p, Kar2p, and Sls1p were revealed using specific polyclonal antibodies as primary antibodies and peroxidase-conjugated anti-IgG antibodies as secondary ones. Detection was realized using the ECL method (Amersham Pharmacia Biotech).

RESULTS

Microsomes Solubilization and Translocation Pores Fractionation—To gain insights about the nature of the translocation sites in *Yarrowia lipolytica*, we checked for the distribution of the major component of the translocation pore, Sec61p, between ribosome-bound and free subcomplexes in comparison with *S. cerevisiae* (8). The first step of this fractionation experiment consists in solubilization of *Y. lipolytica* rough microsomes by detergent. For this purpose, three different detergents were used, and the behavior of YlSec61p as a marker of all translocation pores and of YlSec62p as a marker of the post-translational translocation complex was followed using specific antibodies. A membrane-enriched pellet resuspended in 800 mM KAc was treated either with 2% digitonin, or with 2 and 4% Triton X-100, or with 1 and 2% α-octoglucoside. After sedimentation of ribosomes (P fraction), supernatants corresponding to solubilized extracts were incubated in the presence of concanavalin A-Sepharose, which retains glycosylated proteins (CA fraction), and unbound material was then trichloroacetic acid-precipitated (U fraction). Solubilization of *S. cerevisiae* microsomes was performed as described in Görlich and Rapoport (4). No YlSec61p and only small amounts of YlSec62p were released from the ribosomal pellet using 2% digitonin, whereas about 70% of ScSec61p was solubilized under these conditions (data not shown). Microsomes were also not solubilized using α-octoglucoside. However, as shown in Fig. 1*A*, 4% Triton X-100 led to efficient solubilization of membrane proteins because almost all YlSec62p was present in the supernatant fraction (*lanes 1* and *2*). About 80% of solubilized YlSec62p sedimented with concanavalin A beads (*lane 2* compared with *lane 1*), suggesting that this YlSec62p population belongs to a membrane subcomplex containing at least one glycosylated protein. Under these conditions, only 25% of YlSec61p was released from the ribosomal pellet (*lane 2* compared with *lane 3*), and all of the solubilized YlSec61p was bound by concanavalin A beads (*lane 2* compared with *lane 1*). In contrast, only 50% of ribosome-free ScSec61p, which represents two-thirds of the total ScSec61p population, binds to the lectin (data not shown). The solubilization efficiency and YlSec61p distribution were almost the same using a Triton X-100 concentration of 2% and a KAc concentration of 400 mM (data not shown). To confirm that YlSec61p remaining in the pellet fraction corresponded to ribosome-bound material, puromycin was added to this fraction and salt concentration was increased to release the RAMPs (4, 8). In this experiment, microsomes were first solubilized using 4% Triton X-100 in 400 mM KAc, and salt concentration was then shifted to 800 mM. Concanavalin A-Sepharose was added to the supernatant fraction of a 100,000 rpm centrifugation. As shown in Fig. 1*B*, YlSec61p was absent from the pellet fraction after separation of the ribosomal subunits (*lane 3* compared with *2*), and all released YlSec61p sedimented with the lectin

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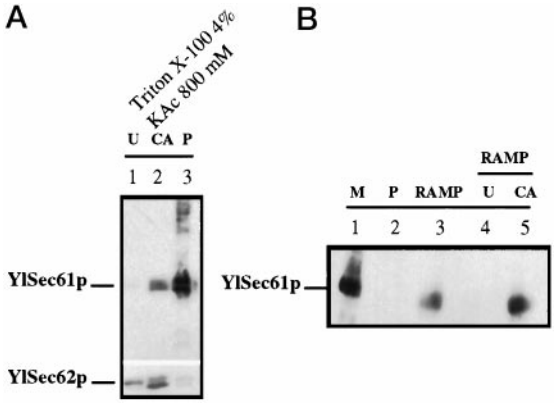

FIG. 1. **Solubilization and fractionation of *Y. lipolytica* endoplasmic reticulum membrane protein.** *A*, solubilization of *Y. lipolytica* microsomes. 20 equivalents were resuspended in a solubilizing buffer containing 50 mM Hepes-KOH, pH 7.5, 800 mM potassium acetate, 16 mM magnesium acetate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 4% Triton X-100. After a 30-min incubation on ice, the sample was centrifuged for 40 min at 70,000 rpm. Supernatant fractions were incubated in the presence of 50 microliters of concanavalin A-Sepharose. Trichloroacetic acid-precipitated unbound material (*lane 1*), lectin-bound material (*lane 2*), and pellet fractions (*lane 3*) were incubated in sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 3% SDS, 0.02% Bromphenol blue) for 20 min at 55 °C before SDS-PAGE analysis and blotting using specific polyclonal anti-Sec61p and Sec62p antibodies. *B*, fractionation of the ribosome-associated membrane proteins. A ribosomal pellet was resuspended in a high salt buffer containing 2 mM puromycin and 0.2 mM GTP and incubated for 30 min on ice and then for 30 min at 28 °C before a 1-h centrifugation at 100,000 rpm yielding a pellet fraction (*lane 2*) and the RAMP fraction (*lane 3*). The latter fraction was incubated with concanavalin A-Sepharose, and bound (*lane 4*) and unbound material (*lane 5*) were analyzed by SDS-PAGE. Proteins were immunoblotted with anti-Sec61p antibodies. *Lane 1*, crude microsomes.

(*lane 5* compared with *4*). About 75% of Sec61p thus co-fractionates with ribosomes in *Y. lipolytica* in contrast with *S. cerevisiae*, where only 30% of Sec61p was present in the RAMP fraction (data not shown).

Sls1p and Kar2p Associate with Sec61p in the Ribosomal Pellet—Since Sls1p and Sec61p were previously shown to co-precipitate (18), the distribution of Sls1p in the same fractionation procedure was tested using specific antibodies. We also checked for the presence in the different fractions of the major lumenal protein Kar2p that was shown to participate in the translocation processes in *S. cerevisiae* (8, 21) and to seal the translocation pore at an early step of co-translational translocation (22). Microsomes solubilization was performed in the presence of 2% Triton X-100 in 400 mM KAc, unsolubilized material was discarded after a low speed centrifugation, and supernatant was then submitted to a 70,000 rpm centrifugation, yielding a high-speed supernatant and a ribosomal pellet. Each fraction was then incubated in the presence of either concanavalin A-Sepharose or anti-YlSec61p antibodies coupled to protein A-Sepharose. While the majority of these two ER lumenal proteins was recovered in the supernatant fraction as expected for soluble proteins, significant amounts of the two proteins were also detected in the ribosomal pellet (Fig. 2*A*, *lane 1*). The following data strongly suggest that these two polypeptides are specifically associated to the ribosome-linked YlSec61p population and do not represent unspecific contamination. First, 40 to 50% of Sls1p and Kar2p present in the pellet fraction were bound by concanavalin A-Sepharose (*lane 2*). Because neither Sls1p nor Kar2p are glycosylated (15), this suggests that they belong to a complex containing at least one glycosylated partner. Second, both were co-immunoprecipitated with YlSec61p (*lane 3*). Third, the ratio of the two proteins in these two samples and in the ribosomal fraction was

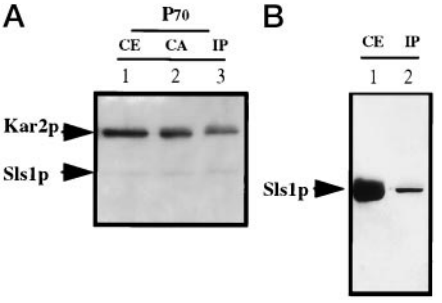

FIG. 2. **Kar2p and Sls1p association.** *A*, 20 equivalents of *Y. lipolytica* microsomes were solubilized using 2% Triton X-100 in a potassium acetate concentration of 400 mM. Supernatant from a low speed centrifugation was then submitted to a 70,000 rpm centrifugation yielding a ribosomal pellet (*lane 1*). Pellet samples were incubated either in the presence of 50 microliters of concanavalin A-Sepharose (*lane 2*) or 100 microliters of anti-YlSec61p antibodies (*lane 3*). Precipitates were eluted in sample buffer for SDS-PAGE analysis. Proteins were immunoblotted with anti-Sls1p and Kar2p antibodies. *B*, anti-Kar2p antibodies were added to solubilized material from a microsomal extract. Kar2p immunoprecipitates were then resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-Sls1p antibodies (*lane 2*) and compared with Sls1p detected in crude extract (*lane 1*).

conserved (*lanes 2* and *3* compared with *lane 1*). Sls1p and Kar2p association with the ribosome-linked Sec61p subcomplex in *Y. lipolytica* argues for a participation in the co-translational translocation process. A co-immunoprecipitation experiment was then performed to confirm that Sls1p and the chaperone protein Kar2p belong to the same sub-complex. Anti-Kar2p antibodies were added to a microsomal extract, and immunoprecipitates were resolved on SDS-PAGE and blotted with polyclonal anti-Sls1p. As shown in Fig. 2*B*, a Sls1p signal was detected in the Kar2p-immunoprecipitate (*lane 2*). This signal was absent when precipitation was performed with the pre-immune serum (data not shown). To address the question of whether the two proteins directly interacted *in vivo*, we used the *S. cerevisiae* two-hybrid system (23). Kar2p and Sls1p coding sequences were cloned in-frame in each of the two vectors. Results are presented in Fig. 3. While all controls were negative (sectors 1, 2, and 3), co-expression of the GAL4-BD-Sls1p and GAL4-AD-Kar2p (sector 4) gave a positive result, allowing transformants to grow on medium devoid of adenine (Fig. 3*B*) or on medium devoid of histidine in the presence of 3-aminotriazole (Fig. 3*C*) and to increase β-galactosidase expression 15-fold (data not shown).

Temperature-sensitive Growth Phenotype Complementing Activity of Sls1p Mutant Proteins—To define Sls1p domains involved in partner binding and to gain insights into Sls1p function, we constructed mutant proteins by site-directed mutagenesis of the *SLS1* gene. Research of an open reading frame sharing some homology with *Y. lipolytica* Sls1p in the *S. cerevisiae* genome data base revealed one putative homolog that displays 52% similarity and 27.5% identity and contains the two N- and C-terminal ER localization signals (Fig. 4). Four mutations (1, 2, 4, and 5) were designed in which the most conserved regions between the *Y.l* product and the *S. cerevisiae* protein were deleted (see Table I and Fig. 4). Mutation 6 consists of substitution of the arginine at position 222. To test the effects of these mutations, the five mutated sequences were integrated at the *LEU2* locus of the Δ*sls1* strain. Because the null strain was previously shown to display a strong temperature-sensitive growth phenotype (18), Leu^+ transformants were grown on YPD plates at 32 °C and compared with a wild-type transformant. Only *sls1*−4 clones were unable to grow at this temperature. All other mutated alleles restored a wild type growth (data not shown). To address the question of whether equal amounts of the mutant proteins were made in

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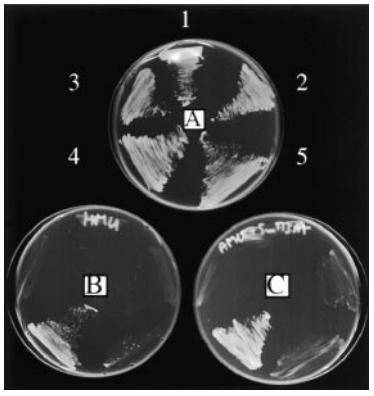

FIG. 3. **Kar2p and Sls1p interaction in the yeast two-hybrid system.** PJ69−4A strain was co-transformed with the following plasmid combinations: *1*, pAS2ΔΔ and pACT2; *2*, pAS2ΔΔ and pACT2-Kar2p; *3*, pAS2ΔΔ-Sls1p and pACT2; *4*, pAS2ΔΔ-Sls1p and pACT2-Kar2p; *5*, pAS2ΔΔ-Sls1−5p and pACT2-Kar2p, and then plated on minimal medium lacking leucine and tryptophan (*A*); minimal medium lacking leucine, tryptophan, and adenine (*B*) and minimal medium lacking leucine, tryptophan, and histidine with 5 mM 3-aminotriazole (*C*).

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Y. l   1 MKFSKTLLLALVAGALAK...........GEDEICRVEKNSGKEI..... 34
         ::: ..:| || :  :|.           :::||...|. .: ||
S. c   2 VRILPIILSALSSKLVASTILHSSIHSVPSGGEIISAEDLKELEISGNSI 51
                 1                                  2
      35 .....CYPKVFVPTEEWQVVWPDQVIPAGLHVRMDYENGVKEAKINDPNE 79
               ||||:| | ::||.::|:| :|:||::|:: :.|:||||:||.::
      52 CVDNRCYPKIFEPRHDWQPILPGQELPGGLDIRINMDTGLKEAKLNDEKN 101

      80 EVEGVAVAVGEEVPEGEVV.IEDLTEENGDEGISANEKVQRAIEKAIKEK 128
                  ||::.::: :|  ||:... || ::|::          :||.
     102 ........VGDNGSHELIVSSEDMKASPGDYEFSSD..........FKEM 133

     129 RIKEGHKPNPNIPESDHQTFSDAVAALRDYKVNGQAAMLPIALSQLEELS 178
         |         || :|:.   |:.:| | |              ::.:: |::
     134 R.........NIIDSNPTLSSQDIARLED............SFDRIMEFA 162
                                                   6 4
     179 HEIDFGIALSDVDPLNALLQILEDAKVDVESKIMAARTIGASLRNNPHAL 228
         |:.. |. : . : :. | ::  :.::.:. : :::|.|:::|||||..:
     163 HDYKHGYKIITHE.FALLANLSLNENLPLTLRELSTRVITSCLRNNPPVV 211

     229 DKVINSKVDL...VKSLLDDLAQSSKEKADKLSSSLVYALSAVLKTPETV 275
         : : :| .::   :.: |.:|.:|.. .:: | ...:  |..:  |:|.:
     212 EFINESFPNFKSKIMAALSNLNDSNHRSSNILIKRYLSILNELPVTSEDL 261

     276 TRFVDLHGGDTLRQLYETGSDD..VKGRVSTLIEDVLATPDLHNDFSSIK 323
         . : .:    .|:::|| :..|  :: :| .||..:| :. .:|| ..:
     262 PIYSTV....VLQNVYERNNKDKQLQIKVLELISKILKADMYENDDTNL. 306

     324 GAVKKRSANWWEDELKEWSGVFQRSLPSKLSSKVKSK.VYTSLAAIRRNF 372
           : ||.|: |..:|:||:: ||  :..|  ..:..: .:..| .::: |
     307 .ILFKRNAENWSSNLQEWANEFQEMVQNKSIDELHTRTFFDTLYNLKKIF 355
                 5
     373 RESVDVSEEFLEWLDHPKKA...........AAEIGDDLVKLIKQDRGEL 411
         :..:.:..:||:||.:. ||            .. .|.: | : :.|  :
     356 KSDITINKGFLNWLAQQCKARQSNLDNGLQERDTEQDSFDKKLIDSRHLI 405

     412 WGNAKARKYDA.RDEL 426
         :||:.|::... ||||
     406 FGNPMAHRIKNFRDEL 421
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FIG. 4. **Alignment of *Y. lipolytica* Sls1p with its *S. cerevisiae* homolog (YOLO31C).** Substituted and deleted amino acid residues are *overlined*.

each strain, an Sls1p-immunoblot analysis was performed. Although the same amount of total intracellular protein was applied on the Western blot, no signal corresponding to Sls1−4p was detected (data not shown). Deletion of the LRNNP sequence thus appears to confer unstability to the mutant protein. Substitution of the conserved basic residue of this peptide for a neutral one had no effect on the protein stability since Sls1−6p was as abundant as the wild type protein and conferred no visible phenotype (data not shown).

Secretion Phenotype Displayed by sls1 Mutants and Kar2p-binding Property of Sls1p Mutant Proteins—To further characterize *sls1* mutants, their secretion phenotype was studied. In a first approach, synthesis and/or secretion of an active alkaline extracellular protease (AEP) was estimated on skim milk plates. After a 48-h incubation at 30 °C, halos were formed around the growing colonies when all mutated alleles, except *sls1*−4 and *sls1*−5, were expressed. A small area of hydrolysis was detected around the colonies of these two mutants after an additional 24-h incubation at the semi-permissive temperature of 28 °C (data not shown). To know if the deficit of protease activity in the *sls1*−5 mutant strain was because of a defect in AEP translocation, a pulse-chase labeling experiment was performed after a 10-min shift at 30 °C (18). The kinetic of appearance of the AEP intracellular mature form in this strain was similar to that of the wild type strain as shown in Fig. 5, indicating that translocated polypeptides are efficiently processed in the *sls1*−5 mutant. AEP intracellular precursors were detected immediately for the two strains. The only difference resides in the amount of newly synthesized AEP precursors: about two-fold less counts were present in the precursor form in an *sls1*−5 context compared with the value obtained for the *SLS1* strain (Fig. 5) while incorporation in total protein was similar. This result indicates that the translocation rate of this co-translationally translocated reporter protein is decreased in the mutant strain. To correlate the secretion phenotype conferred by the Sls1−5p mutant protein to a potential modification of its capacity to interact with the chaperone protein, a co-immunoprecipitation experiment was performed (Fig. 6). While Kar2p amounts were similar in the two strains (*lanes 1* and *2*), no significant amount of Sls1−5p co-precipitating material was detected in the Kar2p immunoprecipitate compared with the wild type protein (*lane 6* compared with *lane 4*). To confirm this first result, the mutated sequence was cloned in the two-hybrid plasmid pAS2ΔΔ and tested against the GAL4-AD-Kar2p fusion protein. No association of the Sls1−5p mutant protein with Kar2p was detected in this test as shown in Fig. 3 (*sector 5*), although expression levels of the fusion protein were similar to that of the wild type Sls1p hybrid product (data not shown). Moreover, the Kar2p-binding property of other mutant proteins, which fully complemented growth and secretion defects of the null strain, was detected using this *in vivo* method (data not shown).

DISCUSSION

We defined solubilization conditions for *Y. lipolytica* microsomes that allowed us in a subsequent fractionation experiment to show that the major translocation pore component, Sec61p, distributes between two sub-complexes: one not linked to ribosomes and the other associated to membrane-bound ribosomes as in *S. cerevisiae* (8). The two yeasts differ in the respective ratio of these two populations. Indeed, from 70 to 80% of Sec61p is present in the RAMP fraction in *Y. lipolytica*, whereas this ratio drops to 30% in *S. cerevisiae*. Sec62p, which belongs to the membrane protein complex required for post-translational translocation (8), is preferentially recovered in the first subcomplex and is absent from the RAMP fraction, indicating that this fraction contains translocation complex involved in co-translational translocation events. In these conditions, small amounts of the chaperone protein Kar2p co-fractionate with Sec61p in the ribosomal pellet, suggesting that the two proteins are associated in the same sub-complex. Accordingly, Kar2p present in this fraction is co-immunoprecipitated by Sec61p antibodies. Involvement of Kar2p in co-translational translocation was already described in the yeast *S. cerevisiae* (21), and the chaperone protein functions as a mammalian translocation pore gate (22). We also checked the behavior of a second lumenal protein identified in *Y. lipolytica*, Sls1p, and showed that minor quantities of this protein are also associated to the ribosome-linked Sec61p population. Using the two-hybrid system, we then showed that Sls1p directly interacts with Kar2p and thus constitutes a new partner of this

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TABLE I
Construction of five mutated versions of Sls 1p

Protein	Oligonucleotide	Deleted or substituted amino acids	Added amino acids
Sls1–1p	**CCGGCAAGGAAATCT**CGCGA**GTCCCTACCGAGGAG**	From 35 to 40	S, R
Sls1–2p	**GGACTATGAAAACGGCG**ATAT**CCCTAACGAAGAGGTTGAG**	From 69 to 76	D, I
Sls1–4p	**CCATTGGTGCTTCTGGCCATGCTCTCGACAAG**	From 221 to 225	G
Sls1–5p	**GTCAGCGAAGAGT**ACT**ACCACCCCAAGAAGG**	From 382 to 387	Y, Y
Sls1–6p	**GGTGCTTCTCTAGGAAACAACCC**	222	G

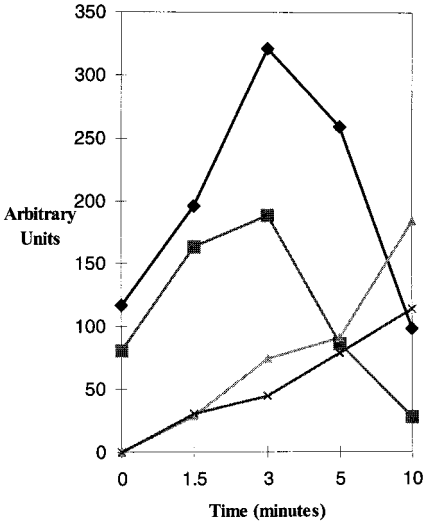

FIG. 5. **Secretion phenotype displayed by the *sls1*−5 mutant.** Radioactive counts measured for the wild type and *sls1*−5 strains in the two AEP intracellular forms at the different time points of the pulse-chase labeling experiment. *SLS1* and *sls1*−5 strains were grown overnight in inducible medium, concentrated, and transferred at 30 °C for 10 min before the 45 s labeling in the presence of a [^{35}S]methionine and cysteine mix. Chase was performed by addition of an excess of cold methionine and cysteine, and samples were taken immediately and 1.5, 3, 5, and 10 min after. Cells were sedimented, and intracellular proteins were immunoprecipitated by anti-AEP antibodies. Immunoprecipitates were resolved on a denaturant 12% polyacrylamide gel and then dried and fluorographed. *Solid diamond*, *SLS1*-pAEP; *gray box*, *sls1*-5-pAEP; *gray diamond*, *SLS1*-mAEP; ×, *sls1*-5-mAEP.

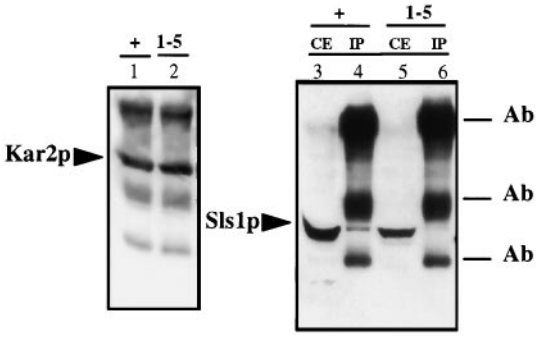

FIG. 6. **Kar2p-binding property of the Sls1−5p mutant protein.** 200 microliters of solubilized extracts from *SLS1* and *sls1*−5 strains were diluted in phosphate buffer saline plus 1 mM phenylmethylsulfonyl fluoride for immunoprecipitation in the presence of anti-Kar2p antibodies and protein A-Sepharose for 4 h at 4 °C. After washes, immunoprecipitates (*IP*) were eluted at 65 °C in sample buffer and resolved on SDS-PAGE with crude extracts (*CE*). Proteins were then transferred to nitrocellulose membranes and blotted with anti-Kar2p (*lanes 1* and *2*) and anti-Sls1p antibodies (*lanes 3* to *6*). *Lanes 1*, *3,* and *4, SLS1* strain; *lanes 2*, *5*, and *6, sls1*−5 strain; Ab, antibodies chains.

chaperone protein.

A mutational analysis then allowed us to conclude that the peptide sequence from amino acid residues 382 to 387 is required for Sls1p function in co-translational translocation and for Sls1p interaction with the chaperone protein Kar2p. The decrease of the co-translational translocation rate in a *sls1*−5 context is not linked to a temperature-sensitive growth phenotype as in the Δ*sls1* strain, suggesting that the Sls1−5 protein still retains a partial function, which is independent of Kar2p-binding. Accordingly, the delay in the secretion of the alkaline extracellular protease and the induction of Kar2p expression observed in the absence of Sls1p (18) were not further detected in a *sls1*−5 context. Studying the mutant and the protein characteristics allows us to propose a model for Sls1p function in the translocation process: Sls1p could first bind to the incoming polypeptide at the lumenal side of the translocation pore. The preprotein could then be directly transferred to the chaperone protein, Kar2p that is recruited to the translocation site through its association with Sls1p, facilitating in that way the crossing of the preprotein. Alternatively, Sls1p could be anchored to the translocation complex through its interaction with Kar2p that thus first binds to the preprotein and then transfers it to Sls1p. Sls1p and Kar2p could act as molecular ratchets as has been proposed for Tim44 and mHsp70 (13). Such an active mechanism could concern a subset of co-translationally translocated secretory proteins; for the majority of them, elongation on the ribosomes may be sufficient to ensure their transfer across the ER membrane (24). In the presence of Sls1−5p, the transfer of the incoming preprotein from one partner to the other would be delayed because Sls1p-Kar2p interaction is inhibited. This study thus provides evidence of Sls1p involvement in the co-translational translocation process that relies on its association with Kar2p.

Sls1p does not contain the 70-amino acid residues DnaJ-like domain present in Sec63p, which represents the main partner of Kar2p in the yeast ER membrane (11). However, the fact that Tim44 displays a very short domain of homology with the Sec63 DnaJ-like domain, and that Tim17 shares no homology at all with it (13), indicate that co-factors of the Hsp70 protein family in translocation are not restricted to the DnaJ protein family. Moreover, because the Sls1p-Kar2p complex does not exist at a 1:1 ratio, we can speculate that Kar2p binds to at least a second partner in the translocation complex. This partner could be a homolog of the *S. cerevisiae* Sec63p as the two proteins were shown to be involved in co-translational translocation (21) or another membrane protein with regard to Kar2p function in sealing the translocation pore at an early stage of the co-translational translocation process (22). Interaction of Sls1p with secretory protein and dependence on ATP of the Sls1p-Kar2p complex function in translocation will be tested in an *in vitro* translocation system.

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