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

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secretory protein translocation. One of these mutant proteins, Sla1p–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 pNA1077 contains a 2.2-kb ClaI–SalI fragment comprising the entire SSL1 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 ClaI–SalI fragments, containing the deletions, were subcloned in a Y. lipolytica integrative plasmid and integrated in the genome of the Sla1 strain previously constructed. MatB, scr1::ADE1, SCR2, his-1, leu2, ura3, sla1::URA3 (18).

Plasmid pAS2DA (TRP1, Amp') was used for expression of GAL4-DNA binding domain fusion proteins and plasmid pACT2 (LEU2, Amp') for expression of GAL4-activating domain fusion proteins. The S. cerevisiae strain JFY6–4A (MATa, trp1–901, leu2–3, 112, ura3–52, his3–200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3, GAL2-ADE2, mat2Δ6::kanMX4) was used for the two-hybrid analysis (20). Two plasmids of p413 transformants were designed to amplify the KAR2 nucleotide sequence between codon 27 and codon 656 (Kar2p-1: 5′-CCG GCC ATG GGC GTT CAG GCT GAT GAT GTG, and Kar2p-2: 4′-5′GGG GAT CCC ACC GTC GTT GGA CTC GTC TC) and the SSL1 coding sequence from codon 19 to codon 411 (Sla1-1: 5′-CCG GCC ATG GGC GAG GAA ATC TGC AGA and Sla1-2: 5′-GGG GAT CCC AAT CCA TAA CTC GCC TCG GTC CTG) After amplification, polymerase chain reaction products were digested by NcoI and BamHI to allow in-frame cloning in the two-hybrid vectors multicloning site. Empty and recombiant two-hybrid vectors were co-transformed into yeast cells by the lithium acetate method. Integrative plasmid and integrated in the genome of a strain previously constructed: sls1::URA3, I-fragments, containing the deletions, were subcloned on minimal medium supplemented with histidine, uracil, and adenine for selection of the plasmids and on minimal medium supplemented with histidine, methionine, uracil, and adenine, or on minimal medium supplemented with histidine, methionine, and uracil plus 5 μm 3-amino-triazole 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 exponential phase cells of S. cerevisiae Sec62p antibodies. ScSec62p and antibodies were kind gifts of R. Schekman and 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 10,000 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 membrane after the migration. Sec61p, Sec62p, Kar2p, and Sla1p 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 (Amer sham 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% octaglcucoside. 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-ac 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 Sec661p was solubilized under these conditions (data not shown). Microsomes were also not solubilized using a-octaglcucoside. However, as shown in Fig. 1A, 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 YlSec62p antibodies 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. 1B, 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.
analyzed by SDS-PAGE. Proteins were immunoblotted with anti-
A-Sepharose, and bound (tated with YlSec61p (glycosylated partner. Second, both were co-immunopreci-
pitation with ribosomes in
50 microliters of concanavalin A-Sepharose. While the majority 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 transfromants to grow on medium devoid of adenine (Fig. 3B) or on medium devoid of histidine in the presence of 3-amin-
triazole (Fig. 3C) 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 mu-
tations (1, 2, 4, and 5) were designed in which the most
conserved regions between the Y. lipolytica and S. cerevisiae sequences were cloned in-frame in each of the two vectors.

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 mu-
tations, the five mutated sequences were integrated at the LEU2 locus of the Y. lipolytica 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 (8, 21) and to seal the translocation pore at an early step of co-translational translo-
cation (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 luminal 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. 2A, 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 were present in the pellet fraction, while the supernatant fraction was 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 conserved (lanes 2 and 3 compared with lane 1). Sls1p and Kar2p association with the ribosome-linked Sec61p subcom-
plex in Y. lipolytica argues for a participation in the co-translational translocation process. A co-immunoprecipitation ex-
periment 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. 2B, 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 transfromants to grow on medium devoid of adenine (Fig. 3B) or on medium devoid of histidine in the presence of 3-amin-
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tations, the five mutated sequences were integrated at the LEU2 locus of the Y. lipolytica 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 Δsls1 strain. Because the null strain was previously shown to display a strong tempera-
ture-sensitive growth phenotype (18), Leu" transfromants 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
Fig. 3. Kar2p and Sls1p interaction in the yeast two-hybrid system. PJ69–4A 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-Sls1p 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-amino triazole (C).

Fig. 4. Alignment of Y. lipolytica Sls1p with its S. cerevisiae homolog (YLO301C). 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 LRNPP sequence thus appears to confer unstability to the mutant protein. Substitution of the conserved basic residue of LRNNP sequence thus appears to confer unstability to the mutant protein. Substitution of the conserved basic residue of LRNNP sequence thus appears to confer unstability to the mutant protein. Substitution of the conserved basic residue of LRNNP sequence thus appears to confer unstability to the mutant protein.

Sls1 Function in Translocation

We defined solubilization conditions for Y. lipolytica microsomes that allowed us in a subsequent fractionation experiment to show that the major translocation pore component, Kar2p, is co-immunoprecipitated along with the chaperone, Sls1p, and shows 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 and Kar2p are associated in the same sub-complex. Accordingly, Kar2p present in this fraction is co-immunoprecipitated 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 with Sec61p in the ribosomal pellet, suggesting that the two proteins are associated in the same sub-complex.

Discussion

We have used Sls1p in its 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, 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 with 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 luminal protein identified in Y. lipolytica, Sls1p, and showed that minor quantities of this protein are also associated to the ribosome-linked Sec61p population.
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 Δsls1 strain, suggesting that the Sls1p–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 luminal 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 sealing the translocation pore will be tested in an in vitro translocation system.

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**TABLE I**

| Protein   | Deleted or substituted amino acids | Added amino acids |
|-----------|-----------------------------------|------------------|
| Sls1–1p   | CCCTCTCTCCCTGCACCTACCTACCTAAGTGGAGGAG | From 35 to 40   |
| Sls1–2p   | CCCTCTCTCCCTGCACCTACCTACCTAAGTGGAG  | S, R             |
| Sls1–4p   | CCATTGGTGCTTCTGGCCATGCTCTCGACACAG    | From 69 to 76   |
| Sls1–5p   | GGACTATGAAAACGGCG                        | D, I             |
| Sls1–6p   | GGACTATGAAAACGGCG                        | From 221 to 225 |

**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 30 min before the 45 s labeling in the presence of a [35S]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 washing, 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
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