The tsr1-1 mutation has been initially identified as an extragenic suppressor of the scr2.11-13 mutation that alters the 7S1L RNA component of the signal recognition particle (SRP) and results in severe defects in protein translocation and SRP stability. We showed previously that the TSRI gene was essential and that the tsr1-1 mutation allowed complete recovery of scr2.11-13-associated secretory defects. We show here that the tsr1-1 mutation also restores SRP stability in an scr2.11-13 context. The TSRI gene product (Tsr1p) is stably associated with rapidly sedimenting material and cofractionates with the lumenal protein Kar2p of the endoplasmic reticulum; it behaves in protease protection assays as a transmembrane component. Communoprecipitation experiments revealed a physical interaction with Kar2p and with ribosomal components associated to the 5.8S rRNA as well as with SRP components like Sec65p and 7S1L RNA. We propose that Tsr1p is an important component of the endoplasmic reticulum membrane, interacting both with the SRP-ribosome complex in the cytosol and with Kar2p in the lumen of the endoplasmic reticulum.

Secretry and membrane proteins in eukaryotic cells are synthesized on ribosomes attached to the endoplasmic reticulum (ER) membrane and targeted by means of the signal sequence from the SRP and its insertion into the ER lumen and thus could play a crucial role in the translocation pathway (11). The overall rate and selectivity of protein translocation across the ER suggest the presence of many interactions with different components of the translocation system including Sec61p complex (5, 6) and other proteins that interact either directly or are in close proximity to the newly synthesized secretory proteins, such as signal peptidase (7), glycosyltransferase (8), protein-disulfide isomerase (9), and BiP (10). Other interactions with the ribosome seem to be required for the direct transfer of the nascent chain to the protein-conducting channel. The membrane anchoring of ribosomes engaged in protein translocation is mediated by the Sec61p complex (11).

In Yarrowia lipolytica, substitutions at the G128 and A130 positions of the stem loop 1 of 7S1L RNA, which forms the presumed binding site of Srp19p (12), have permitted selection of a thermosensitive mutant scr2.11-13. Defects in this mutant include a decrease in the synthesis of secretory proteins, preferential cleavage of the 7S1L RNA, and a substantial instability of the SRP. We have reported the isolation of a suppressor of the scr2.11-13 mutation (13). This suppressor, called tsr-1 (thermosensitive rescued), restores normal growth to scr2.11-13 mutants (tsr1-1(scr2.11-13)) at 34 °C but not to a wild type (SCR2) strain (tsr1-1(SCR2)). Complementation of the tsr1-1 thermosensitive phenotype has made it possible to isolate the TSRI gene that encodes a serine-threonine-rich protein of 461 amino acids with an amino-terminal leader sequence and a transmembrane domain.

The present report describes the stability of the SRP in the tsr1-1(scr2.11-13) context and addresses the features of the TSRI gene product (Tsr1p) and its relation to the SRP and translocation apparatus. We find that Tsr1p is an ER membrane protein that interacts with the SRP in the cytosol and BiP in the ER lumen and thus could play a crucial role in the translocation pathway either as a component of the translocon or as a functional intermediate between the lumen and the cytosolic side of the ER.

EXPERIMENTAL PROCEDURES

**Strains, Growth Conditions, and Materials**—Strains and plasmids used in this study are listed in Table I. The YPD medium contained 1% yeast extract, 1% Bacto-Peptone, 1% glucose, and 2% agar. The selective minimal medium contained 1% glucose, 0.17% yeast nitrogen base without ammonium sulfate (Difco, Detroit, MI), and 0.1% proline as nitrogen source and was supplemented with appropriate nutrients.

All enzyme reactions, DNA, and RNA extractions, and hybridization preparations were performed as described by Maniatis et al. (14). Transformation of Y. lipolytica was performed as described by Xuan et al. (15). Dithiobis succinimidylpropionate (DSP) was from Sigma.

**Protein Immunoblotting and Immunoprecipitation**—Protein extracts were prepared from yeast cells, separated through 10% SDS-polyacrylamide gels, blotted to nitrocellulose, and probed with specific antibody. Bound antibodies were visualized by enhanced chemiluminescence (Amersham Corp.). Native immunoprecipitation of the SRP was performed as described by He et al. (12).

The protocol of coimmunoprecipitation described by Enzaut et al. (32)
was adapted for the immunoprecipitation of the 7SL RNA, Kar2p, and Sec65p by the anti-GST-Tsr1p antibody. Cell fractionation was performed as described by Ruohola and Ferro-Novick (17).

Anti-Tsr1p Antibody Preparation—The pML5 plasmid containing the GST-TSR1 fusion has been obtained by cloning the BclI-EcoRI fragment carrying the TSR1 gene downstream from the GST open reading frame of the pGEX 2T vector (Stratagene). The GST-Tsr1p fusion protein was overexpressed, purified on a GST-Sepharose column (Pharmacia Biotech Inc.) and used to immunize rabbits. The sera were tested and used at 1:10,000 dilution for immunoblots. In pML5 containing Escherichia coli cells, these antibodies recognized a single protein with the mobility expected for the GST-Tsr1p fusion.

RESULTS

The tsr1-1 Mutation Promotes a Complete Recovery of the SRP Stability Defect of scr2.II-13—It has been reported using the human autoimmune anti-SRP antibody that the stability of the SRP was greatly reduced in the scr2.II-13 mutant (12). To assess whether the suppressive mutation tsr1-1 could repair this instability, cell lysates from scr2.II-13, tsr1-1 (scr2.II-13), and wild type strains were immunoprecipitated with an anti-Sec65p antibody. Immunoprecipitates were analyzed on 6% polyacrylamide, 8.3 M urea denaturing gel and hybridized either with the SalI-ClaI fragment of pINA823 containing the SCR2 gene to probe the 7S RNA (A) or the BglII-EcoRI fragment of pINA24 containing the rDNA to probe the 5.8S (B). RNA was then scanned, and the results of the quantification of the 7S RNA (A') and the rRNA 5.8S (B') are reported. Lanes 1 and 4, wild type strain; lanes 2 and 5, scr2.II-13 strain; lanes 3 and 6, tsr1-1(scr2.II-13) strain.

FIG. 1. tsr1-1 stabilizes the SRP. Whole cell lysate was prepared from scr2.II-13, tsr1-1(scr2.II-13), and wild type strains by gentle lysis of spheroplasts and immunoprecipitated with the anti-Sec65p antibody. RNA from the supernatant and the immunoprecipitates (Pellet) was recovered and analyzed on 6% polyacrylamide. 8.3 M urea denaturing gel. RNA was then transferred to Nylon Hybond N membrane and hybridized either with the SalI-ClaI fragment of pINA823 containing the SCR2 gene to probe the 7S RNA (A) or the BglII-EcoRI fragment of pINA24 containing the rDNA to probe the RNA 5.8S (B). RNA was then scanned, and the results of the quantification of the 7S RNA (A') and the rRNA 5.8S (B') are reported. Lanes 1 and 4, wild type strain; lanes 2 and 5, scr2.II-13 strain; lanes 3 and 6, tsr1-1(scr2.II-13) strain.

TABLE I

| Strains/plasmids | Description | Source or reference |
|------------------|-------------|---------------------|
| **Y. lipolytica** | **MatB, hisI-1, leu 2-35, ura 3-302, SCR1:ADE1, Δ SCR2 (LEU2, SCR2)** | Ref. 12 |
| *tsr1–1 (URA3, SCR2*)* | MatB, hisI-1, leu 2-35, ura 3-30, tsr1–1 SCR1:ADE1, Δ SCR2 (URA3, SCR2*) | This work |
| *tsr1–1 (LEU2, scr2.II-13)* | MatB, hisI-1, leu 2-35, ura 3-30, tsr1–1 SCR1:ADE1, Δ SCR2 (LEU2, scr2.II-13) | This work |
| pINA823 | pBR322 carrying Y. lipolytica SCR2 and LEU2 genes | This laboratory |
| pINA24 | pBR322 carrying Y. lipolytica rDNA gene | Ref. 24 |
| pML5 | Derivative of pGEX-2T carrying the GST-TSR1 fusion | This work |
these data we concluded that the suppressive effect of tsr1-1 may result in a tighter association of ribosomes with the mutated SRP.

Membrane Association of Tsr1p—We generated polyclonal antibodies directed against a GST-Tsr1p fusion protein (see "Experimental Procedures"). On a Western blot of total cell extract treated with 4% Triton X-100, (Fig. 2A), the immune serum (lane 2) but not the preimmune serum (lane 1) recognized a 50-kDa protein. To determine the in vivo localization of the Tsr1p, extracts obtained from wild type cells by gentle spheroplast lysis were fractionated by differential centrifugation: 10 min at 10,000 × g followed by 20 min at 30,000 × g. At each stage, the supernatants and the pellets were collected and prepared for SDS-PAGE. Proteins were transferred to a nitrocellulose filter, and the immunoblot was decorated with Tsr1p antibodies. Fig. 2A (lanes 3–6) demonstrates that most of Tsr1p fractionated in the 10,000 × g pellet. The preferential association of Tsr1p with rapidly sedimenting material seems compatible with a mitochondrial or ER localization (18).

Tsr1p Is an ER Transmembrane Protein—The sequence analysis of Tsr1p highlighted a transmembrane domain (13). To confirm that Tsr1p was indeed a membrane-spanning protein, spheroplasts were gently lysed, and cellular membranes were sedimented by a 10,000 × g centrifugation. The resuspended membrane pellet was subjected to a set of treatments followed by a centrifugation at high speed, and evaluation of the supernatant and pellet fractions were analyzed by immunoblot using the anti-GST-Tsr1p polyclonal antibodies. Agents that release peripheral proteins associated with membranes by weak electrostatic bonds (2.5 M urea, 0.5 M acetate, 0.1 M carbonate at pH 11.5) had no effect on the fractionation of Tsr1p. Whereas most cellular protein was found in the fractions F15 and F16, whereas Triton X-100 and Triton X-114 stripped 85–90%. These results indicate that Tsr1p is a transmembrane protein. To examine the topology of Tsr1p in the membrane, the 10,000 × g membrane fraction was subjected to digestion by 0.1 mg/ml proteinase K in the presence or the absence of 4% Triton X-100. After quenching of the reaction with 25% trichloroacetic acid, samples were resolved by a 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the GST-Tsr1p polyclonal antibody. Results in Fig. 3 show that in the absence of detergent, Tsr1p was digested to a 34-kDa resistant fragment, which is compatible with a removal of the Tsr1p carboxyl terminus and suggests that this domain is localized on the cytosolic side. In the presence of 4% Triton X-100, Tsr1p was completely proteolyzed.

To address whether Tsr1p was an ER membrane protein, spheroplasts were gently lysed, and cellular membranes were subfractionated in a sucrose gradient into 22 fractions and analyzed using antibodies raised against Tsr1p and Kar2p. Whereas most cellular protein was found in the fractions F15 and F17 (Fig. 4A), results in Fig. 4B show that Tsr1p and Kar2p were both localized in the fractions F11 and F12. This colocalization between Tsr1p and Kar2p supports the conclusion that Tsr1p is an ER component.

Tsr1p Interacts with the SRP, Ribosomes, and BiP—To confirm the interactions of Tsr1p with the SRP or components of the translocation apparatus, we examined the immunoprecipitation of the 7SL RNA, Sec65p, 5.8S rRNA, and Kar2p using the GST-Tsr1p polyclonal antibody. Cells from wild type strain were grown overnight to early-log phase and 1 A_{590} × ml were collected. Cell lysates were treated or not with DSP and
mg/ml DSP and 4% Triton X-100.

FIG. 4. Colocalization of Tsr1p and Kar2p. Wild type wild strain and cellular membranes were sedimented and subfractionated in a sucrose gradient. A total of 22 fractions, each 500 μl, were collected. A, dosage of whole proteins in each fraction. B, sample from each fraction was heated 5 min at 100 °C and subjected to SDS-PAGE and immunobotted with anti-GST-Tsr1p antibody. Bound antibody was visualized by ECL (Amersham Corp.).

FIG. 5. Coimmunoprecipitation of 7SL RNA and Sec65p with the Tsr1p. A, whole cell lysates were prepared from a wild type strain by gentle lysis of spheroplasts (whole) and immunoprecipitated with the preimmune and the anti-GST-Tsr1p sera. RNA was recovered from the immunoprecipitates and subjected to Northern blot using to the SalI-ClaI fragment of pINA23 containing the SCR2 gene as a probe. pI, preimmune immunoprecipitates; I, immune immunoprecipitates. B, whole cell lysate was prepared from a wild type strain by gentle lysis of spheroplasts, treated or not with DSP as indicated and immunoprecipitated with the preimmune and the anti-GST-Tsr1p serum. Solubilized proteins were recovered from the immunoprecipitates, resolved on 12.5% SDS-PAGE, and immunobotted with anti-Sec65p serum. Bound antibody (Ab) was visualized by ECL (Amersham Corp.). 7SL, preimmune immunoprecipitates; I, immune immunoprecipitates.

immunoprecipitated with the GST-Tsr1p polyclonal antibody. RNAs were recovered from the immunoprecipitates, analyzed on a 6% polyacrylamide, 8.3 M urea denaturing gel and hybridized with an SCR2 probe. Results in Fig. 5A show that the anti-Tsr1p antibody precipitated a small fraction of SRP RNA that is compatible with an interaction between Tsr1p and the SRP. To confirm this interpretation, proteins recovered from the immunoprecipitates were analyzed on 12.5% SDS-PAGE gels, transferred to nitrocellulose filter, and decorated with the Sec65p serum. Results in Fig. 5B show that a significant immunoprecipitation of Sec65p was obtained after treatment with 4% Triton X-100 followed by cross-linking using DSP. This treatment increases the accessibility of DSP to molecular complexes between Tsr1p and other proteins. Comparing the amount of Sec65p in the supernatant and in the immunoprecipitates, 65% of Sec65p has been estimated to interact with Tsr1p (data not shown). Both immunoprecipitation of 7SL RNA and Sec65p indicate clearly that Tsr1p interacts with the SRP. To understand whether this interaction is obtained with free SRP or within a SRP-ribosome complex, we have examined the coimmunoprecipitation of 5.8S rRNA with the Tsr1p. Results in Fig. 6A show that a clear immunoprecipitation of 5.8S rRNA is obtained with the Tsr1p antibodies, suggesting that the Tsr1p interacts with the SRP while coupled to ribosomes. To test whether the Tsr1p could interact through its luminal domain with components of the ER lumen, we have tested the coimmunoprecipitation of Kar2p with the Tsr1p. Results in Fig. 6B show that in the case of Kar2p, coimmunoprecipitation is detected even without DSP treatment.

All these results show that Tsr1p interacts both with the SRP, probably during its targeting as a SRP-ribosome-nascent chain complex to the ER membrane, and with Kar2p. Its membrane localization suggests that it may involve its amino-terminal and its carboxyl-terminal domains, respectively, in each of these interactions.

DISCUSSION

We have previously described the isolation of the tsr1-1 mutation as a suppressor of the thermosensitivity conferred by scr2 II-13, a mutation of the SRP RNA that results in defects in the translocation of secretory proteins and destabilizes the SRP in the yeast Y. lipolytica (13). tsr1-1 restored normal growth to
Ts1p is a serine-threonine-rich protein of 461 amino acids that shows extensive homology with two putative proteins Yhc8p from Saccharomyces cerevisiae and Yhu2p from Hansenula polymorpha (19). The presence of an amino-terminal signal sequence and of a membrane spanning domain suggested that Ts1p was an integral membrane protein of the ER membrane. Immunoblot experiments using a Ts1p antiserum revealed a protein with a molecular mass of ± 50 kDa in close agreement with predictions derived from sequencing data. Using cell fractionation experiments we have demonstrated that Ts1p was associated with a rapidly sedimenting fraction and required treatment with detergents to be solubilized. Proteinase K treatment in the absence of detergent showed that the Ts1p molecular mass shifted from 50 to 34 kDa, which is compatible with the removal of its carboxyl-terminal domain. Fractionation experiments show a significant colocalization between Ts1p and Kar2p. Taken together, these results strongly suggest that Ts1p is a type I transmembrane protein of the ER, exposing its carboxyl-terminal to the cytoplasm and its amino-terminal to the ER lumen.

Genetic and biochemical interactions indicated that Ts1p interacts with components of the SRP and of the translocation apparatus. First, the tsr1-1 mutation was isolated as an allele-specific suppressor of a 7SL RNA mutation scer2.11-13. Second, immunoprecipitation analysis using anti-Sec65p antibodies showed that contrary to a scer2.11-13 mutant, where little 7SL RNA was coimmunoprecipitated with Sec65p, in tsr1-1(scer2.11-13) suppressed cells, the SRP stability was completely restored. Third, the Ts1p antiserum immunoprecipitated, in a wild type strain, the SRP-ribosome complex as judged by the coimmunoprecipitation of Sec65p, rRNA 5.8S, and 7SL RNA. Coimmunoprecipitation by the Ts1p antiserum of Kar2p, an ER luminal chaperone protein from the HSP70 class, was detected even without cross-linking and suggests a strong interaction between these two proteins. This interaction appears very important since recent results have shown that binding of secretory precursor polypeptides to a translocon subcomplex is regulated by BiP (20) and that Kar2p can act in cooperation with some components of the translocon to stimulate translocation by pulling the secretory protein precursors across the ER membrane (21, 22).

Together our results indicate clearly that Ts1p participates in a complex involving these components, and because Ts1p is an essential gene, we have to conclude that it can play an important role in the SRP-dependent translocation events at least.

How does the mutated Ts1p suppress the scer2.11-13 defect? Our results show that it does not restore an almost wild type structure to an SRP containing a defective scer2.11-13 mutant RNA, because this RNA is still as susceptible to degradation as it was in the original Ts1p background. This nuclease cleavage seems to reflect a secondary effect of the mutation, not directly responsible for the secretory defect. We observed that in a tsr1-1(scer2.11-13) strain, a ribosomal component, the 5.8S RNA, was much more efficiently coimmunoprecipitated by antibodies directed against Sec65p, a SRP component, than it was in a Ts1p strain, mutated or not for the 7SL RNA. This strongly suggests that tsr1-1 reinforces or stabilizes interactions between SRP and the ribosome at the docking or at the translocation site. Because the primary defect of scer2.11-13 mutated SRP has been postulated to involve inefficient targeting of this SRP to the ER, it is tempting to speculate that tsr1-1 may suppress scer2.11-13 by increasing the half-life of the ribosome complex at the receptor or translocation site. Conversely, this would account for the deleterious effect of tsr1-1 in an scer2.11-13 context, where the clearance time would be unduly lengthened.

What would then be the function of wild type Ts1p? In the above scheme, Ts1p would be required to accelerate dissociation of the SRP from the ribosome. Recent work from Bacher et al. (23) suggests that ribosomes play a crucial role in the GTPase cycle of the SRP, acting as GTP loading factors for SRP54 in the presence of a signal peptide and strengthening the SRP-ribosome complex. Disassociation of the complex is thought to occur at the SRP receptor, where a GTP-loaded SRP receptor α subunit activates the GTPase activity of SRP54. Activation of SRP receptor α by GTP requires an unknown partner, which may be the translocon or a translocon-associated protein. A likely place for Ts1p in this puzzle would be at this level, where it could gauge through its luminal domain, possibly through Kar2p interaction, the state of clearance of the translocon and control SRP-ribosome dissociation through its cytoplasmic domain. The recent identification of a TSR1 functional homologue in S. cerevisiae2 should permit testing these possibilities.

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