Disruption of YHC8, a Member of the TSR1 Gene Family, Reveals Its Direct Involvement in Yeast Protein Translocation*

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Genetic studies of *Saccharomyces cerevisiae* have identified many components acting to deliver specific proteins to their cellular locations. Genome analysis, however, has indicated that additional genes may also participate in such protein trafficking. The product of the yeast *Yarrowia lipolytica* TSR1 gene promotes the signal recognition particle-dependent translocation of secretory proteins through the endoplasmic reticulum. Here we describe the identification of a new gene family of proteins that is well conserved among different yeast species. The TSR1 genes encode polypeptides that share the same protein domain distribution and, like Tsr1p, may play an important role in the early steps of the signal recognition particle-dependent translocation pathway. We have identified five homologues of the TSR1 gene, four of them from the yeast *Saccharomyces cerevisiae* and the other from *Hansenula polymorpha*.

We generated a null mutation in the *S. cerevisiae* YHC8 gene, the closest homologue to *Y. lipolytica* TSR1, and used different soluble (carboxypeptidase Y, a-factor, invertase) and membrane (dipeptidyl-aminopeptidase) secretory proteins to study its phenotype. A large accumulation of soluble protein precursors was detected in the mutant strain. Immunofluorescence experiments show that Yhc8p is localized in the endoplasmic reticulum. We propose that the YHC8 gene is a new and important component of the *S. cerevisiae* endoplasmic reticulum membrane and that it functions in protein translocation/insertion of secretory proteins through or into this compartment.

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‡ The abbreviations used are: SRP, signal recognition particle; ER, endoplasmic reticulum; PCR, polymerase chain reaction; HA, hemagglutinin; CPY, carboxypeptidase Y; DPAP, dipeptidyl-aminopeptidase.
The TSR1 gene codes for a serine-rich protein of 50 kDa spanning the ER membrane. Genetic and biochemical studies implicated Tsr1p in the stability of SRP in the early steps of the SRP-dependent translocation pathway of secretory proteins. Cross-linking studies revealed that Tsr1p interacts with the ribosome-SRP complex and with BiP (16). Following the sequencing of the S. cerevisiae genome, we identified four homologues of TSR1 in this unrelated fungal species, and we suggest here that they define a new gene family to be called the TSR1 gene family. To assess whether the closest of the TSR1 homologues of YHC8 is also critically involved in protein translocation, we constructed a null mutant allele of this gene and examined the effect of its loss of function in S. cerevisiae.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—Strains and plasmids used in this study are listed in Table I.

**Media**—YPG medium contained 1% yeast extract, 1% Bacto-Peptone, and 2% glucose. YP medium contained 1% yeast extract, 1% Bacto-Peptone, and 0.1% glucose. The selective minimal medium contained 2% glucose. YPinv medium contained 1% yeast extract, 1% Bacto-Peptone, and 0.1% proline as nitrogen source and was supplemented with 2% glucose. YPG medium contained 1% yeast extract, 1% Bacto-Peptone, and 0.1% glucose. The selective minimal medium contained 2% glucose. YPinv medium contained 1% yeast extract, 1% Bacto-Peptone, and 0.1% proline as nitrogen source and was supplemented with 2% glucose.

**DNA Techniques**—All enzyme reactions and DNA preparations were performed as described by Maniatis et al. (17). Oligonucleotides used for PCR disruption of YHC8 gene were as follows: oligonucleotide 1, CGG CCC GCC CCC CAT CGA AGG GGT GCT ACT G; oligonucleotide 2, GCA TAT AAC GCT ACA TAC TAG CC; oligonucleotide 3, TCC ATG ACG GTT GCT ACT G; oligonucleotide 4, GGGA GGG CCC GCC GGG GGT ATA GAC GAT GGC TCT TAT T. Bold characters indicate the sticky end I site (18). The first PCR reactions utilized primers 1 and 2 or 3 and 4. The 1-kilobase pair amplified fragment was tested for the presence of the newly synthesized HindIII site of pINA-KAN (18).

**PCR reactions** were developed as described by Matah and colleagues (18). The first PCR reactions utilized primers 1 and 2 or 3 and 4. A second PCR using the products of the first PCR as a template was performed using oligonucleotides 2 and 3. The 1-kilobase pair amplified fragment was tested for the presence of the newly synthesized AscI site and blunt-ended using the T4 DNA polymerase. The plasmid pSC10 was obtained by integration of the blunt-ended fragment obtained from the second PCR at the PuoI site of pDNA-KAN (18).

**Protein Immunoblotting**—Yeast cultures were grown overnight to early log phase and 2 A600/ml were collected. The cells were washed in 10 mM Na3PO4 and resuspended in 100 μl of 2× SDS-polyacrylamide gel electrophoresis sample buffer containing 1 mM phenylmethylsulfonfyl fluoride. Cells were lysed by vortexing with glass beads (0.5 mm diameter), heated for 10 min at 95 °C, separated on 10% SDS-polyacrylamide gel electrophoresis sample buffer containing 1 mM phenylmethylsulfonfyl fluoride. Cells were lysed by vortexing with glass beads (0.5 mm diameter), heated for 10 min at 95 °C, separated on 10% SDS-polyacrylamide gel electrophoresis sample buffer containing 1 mM phenylmethylsulfonfyl fluoride. Cells were lysed by vortexing with glass beads (0.5 mm diameter), heated for 10 min at 95 °C.

**Immunofluorescence Experiment**—YHC8 open reading frame was amplified using two oligonucleotides carrying flanking BamHI and NotI restriction sites (CGG GAT CCG CAA AAA CGC ATG CAG ACG and CCC GGC GCC GGC GGC GGT CAT TAG, respectively) and cloned into pYEF2 designed by C. Cullin (19). This construction put yhc8 under the control of the GAL10 promoter with the HA tag at the carboxyl-terminal end. In order to exchange the HA tag for the protein A tag, a NotI-Bsu36I fragment was amplified from the p2P plasmid and inserted in the corresponding NotI-Bsu36I. Expression of the tagged Yhc8p under the control of GAL1 promoter was monitored by growing cells to A600 = 1 in synthetic media containing 2% raffinose. Galactose was added to a final concentration of 2%. Cells were collected at 0, 30, 60, and 90 min (20). Fixation and antibody decoration procedures were adapted from Pringle and collaborators (21). The primary antibody was an anti-protein A rabbit (Sigma reference number P-3775) used at a 1:300 dilution. The fluorescent secondary antibody was a Cy3-conjugated anti-rabbit IgG from donkey (reference number 711-165-152, Jackson Immunoresearch, West Grove, PA) at a dilution 1:300. Observations were performed on a Leitz Laborlux S microscope.

**RESULTS**

**TSR1 Gene Family**—We have previously described the in vivo evidence for the role of the ER membrane protein, Tsr1p, in the translocation pathway of the yeast Y. lipolytica (15). A first homology search had identified two homologues, YHC8 from S. cerevisiae and YLU2 from Hansenula polymorpha (23) of the TSR1 gene. A new search through the entire S. cerevisiae genome sequence data base led to the identification, in addition to YHC8, of three other homologues called Hre556, Sycn2283, and UNP378. All of these genes encode putative proteins which, like Tsr1p and Ylu2p, contain an amino-terminal signal sequence and share a highly conserved distribution of 5 domains as follows: cysteine-rich (Cys-rich), serine/threonine-rich (Ser/Thr-rich), intermediary, transmembrane, and cytoplasmic (Fig. 1, A and B) (15). The topology of this last domain was previously provided for Tsr1p (16). Fig. 1 summarizes the features of this gene family that we called the TSR family. In addition to the structural conservation (Fig. 1A), the similarity of the sequences of the four predicted proteins with Tsr1p increases significantly toward the NH2-terminal cysteine-rich domain (results not shown). Comparison of the putative cytosolic domain of the six proteins shows high conservation between Sycn2283 and Hre556p, and Tsr1p and Yhc8p (Fig. 1, C and D). Based on the fact that the Tsr1p was more similar to Yhc8p than to any of the other homologues (Fig. 1, C and D), we focused our study on the YHC8 gene of S. cerevisiae, and we tested its possible involvement in the early steps of the secretory pathway.

The nucleotide sequence of the YHC8 gene revealed an open reading frame of 1815 base pairs. RNA hybridization experiments confirmed that this gene was expressed and produced a
Direct Involvement of YHC8 Gene in Protein Translocation

### A

| Protein   | SP | C-rich | S/T-rich | I | Tm | Cyt | Size (aa) |
|-----------|----|--------|----------|---|----|-----|-----------|
| Tsr1p (Y.l.) |    |        |          |   |    |     | 461       |
| Yhc8p (S.c.)  | 54%| 39%    |          |   |    |     | 605       |
| Hre556p (S.c.) | 32%| 65%    |          |   |    |     | 556       |
| Scynl283p (S.c.) | 20%| 65%    |          |   |    |     | 503       |
| Unf378p (S.c.)  | 33%| 47%    |          |   |    |     | 378       |
| Ylu2p (H.p.)  | 33%| 51%    |          |   |    |     | ?         |

### B

- **Scynl283p**
- **Hre556p**
- **Ylu2p**
- **Yhc8p**
- **Tsr1p**
- **Unf378p**

### C

- **Scynl283p (S.c.)**
- **Hre556p (S.c.)**
- **Ylu2p (H.p.)**
- **Yhc8p (S.c.)**
- **Tsr1p (Y.l.)**
- **Unf378p (S.c.)**

### D

- **Scynl283p (S.c.)**
  - TPA ...
- **Hre556p (S.c.)**
  - TPA ...
- **Ylu2p (H.p.)**
  - TPA ...
- **Yhc8p (S.c.)**
  - TPA ...
- **Tsr1p (Y.l.)**
  - TPA ...
- **Unf378p (S.c.)**
  - TPA ...
The diploid strain FY1679, and Kanr clones were selected. Dis-
then targeted for integration into the kanamycin resistance cassette. The linearized plasmid was
and served to construct the disrupting plasmid pSC10 carrying
(SEP) disruption strategy was adopted (18). The promoter and
package with the scoring matrix of Risler
et al.
D,
program from GCG.

Deletion of YHC8 Induces Defects in the Translocation of Soluble Secretory Proteins—To determine whether YHC8 is
important for protein translocation, we examined the fate of several well characterized secreted proteins in a yhc8::Kanr
strain by immunoblots of whole cell extracts.

Carboxypeptidase Y (CPY) is a vacuolar protease that is
synthesized as a 59-kDa inactive precursor (prepro-CPY). Re-
moval of its amino-terminal signal sequence in the ER lumen
gives rise to the 57-kDa pro-CPY. ER glycosylation of pro-CPY
yields the 67-kDa p1 form (24), which is then transported to the
Golgi apparatus where it is glycosylated to generate the 69-kDa
p2 form. Upon arrival in the vacuole, an amino-terminal sequence
of approximately 8 kDa is removed from the p2 form, yielding
the 61-kDa mature CPY, active form (m-CPY). This
maturation of p2 requires the PEP4 gene product (Ref. 25; see
Fig. 3A). Fig. 3A shows the fate of CPY in Δyhc8, wild type
(SEC+), and sec mutant cells. To discriminate between the
prepro and m-CPY forms all the strains used in the CPY
immunoblots experiments carried the pep4-3 mutation so that
no maturation of the p2 could occur in the vacuole. In
addition, we used the ER-to-Golgi blocked sec18-1 mutant (Fig.
3A, 6th lane) to detect the ER p1 form. Results in Fig. 3 show
that for sec61, -62, and -63 mutants primarily prepro-CPY and
p2 forms were detected and for Δyhc8 mutants primarily pre-
pro-CPY and pro-CPY forms were detected.

The secretory invertase is synthesized as a precursor of 61
kDa with a 19-residue hydrophobic signal sequence (absent
from the cytosolic form) which ensures its targeting to the ER
membrane. Upon translocation to the ER, this precursor under-
goes signal peptide cleavage and core glycosylation (26, 27).
Transported to the Golgi apparatus, it is subjected to further
mannosylations before reaching its peripheral location (28).
To test the effect of the absence of the YHC8 product on
the translocation and transit of the invertase precursors, we com-
pared the secretory phenotype of the Δyhc8 mutant with that of
wild type and sec mutant cells (Fig. 3B). In the wild type cells
primarily highly glycosylated forms were detected (5th lane).
For sec mutants primarily pre-invertase and the highly glyco-
sylated forms (1st to 3rd lanes) were detected; Δyhc8 mutant
cells, however, accumulated preinvertase (4th lane), and only a
small level of highly glycosylated forms can be detected.

The α-factor mating pheromone is a 13-amino acid peptide
that is secreted into the culture medium by MATα cells (29). It
Is synthesized as a precursor polypeptide of 21 kDa (pp-αF)
that contains a prepro-leader sequence of 83 amino acids.
Cleavage of the signal sequence after translocation into the ER
gives rise to the pro-α-factor. This is then decorated with three
core oligosaccharides during its translocation across the ER
membrane, yielding a 26-kDa ER form (30–32). Directed to the
Golgi apparatus, this form undergoes outer chain glycosylation
and proteolytic maturation (33). The processing is then com-
pleted within the secretory vesicles by Kex2p and dipeptidyl-
aminopeptidase A (DPAP A) (34). For Δyhc8 and like sec
mutants (Fig. 3C, 1st to 3rd lanes) the cells accumulate the prepro-
α-factor (4th lane), which indicates a significant defect in the
translocation of this molecule into the ER.

Deletion of YHC8 Induces Defects in the Assembly of Mem-

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**Fig. 1. The TSR1 gene family.** A, structural organization of the five putative homologous proteins encoded by the genes of the TSR1 gene
family. The percentages of identity between Tsr1p and the other five proteins of the TSR1 gene family for the cysteine-rich, serine/threonine-rich,
and cytoplasmic domains are indicated. Ts, transmembrane; Cyt, cytoplasmic; S.c., S. cerevisiae; H.p., H. polymorpha; Y.l., Y. lipolytica; aa, amino
acid. Tsr1p (15), Yhc8p (SwissProt accession number, P38739), Scynl238p (EMBL accession number, Z71559), Hre556p (PIR accession number,
S1892), Ylu2p (23) and Unm378p (GenBank accession number, U39481). B, comparison of the pattern of hydrophobicity of the different members
of the TSR1 gene family, C, a tree of sequence similarities showing the Tsr1p with its homologues. The tree was generated using the Pileup
program from GCG. D, alignment of the cytoplasmic domains of the five homologous proteins using the PILEUP program from the GCG software
package with the scoring matrix of Risler et al. (40). Identities between Scynl238p, Hre556p, Ylu2p, Tsr1p, and Yhc8p are presented in bold.
brane Proteins—To test the effect of YHC8 deletion on the insertion of membrane proteins into the ER membrane, we used the dipeptidyl-aminopeptidase B (DPAP B) as a reporter protein and compared its kinetics of insertion in Dyhc8 and sec mutant strains. DPAP B is an integral membrane glycoprotein with a carboxyl-terminal domain localized in the lumen of the ER (35). The unglycosylated pre-DPAP B can be observed at 96 kDa, and the mature vacuolar form migrates as a 120-kDa species. Contrary to the wild type or sec61-2 mutant strains where no accumulation or only a small accumulation of pre-DPAP B was detected (Fig. 3A, 1st and 5th lanes) (4), immunoblotting from Dyhc8 showed accumulation of pre-DPAP B (4th lane). However, the amount is not as great as that observed in sec62-1 and sec63-1 mutants (2nd and 3rd lanes). These results clearly demonstrate a partial defect in the assembly of this integral membrane protein in the yhc8 null mutant cells.

Yhc8p Is an ER Membrane Protein—In order to localize Yhc8p inside the cell, it was tagged at its carboxyl-terminal end using the vectors developed by Cullin and Minvielle-Sebastia (19) which fused the HA tag and placed the open reading frame under the control of the GAL1 promoter. When grown on 2% galactose as carbon source, cells decorated with anti-HA-α-factor in a Δyhc8-deleted strain. Yeast whole cell extracts were prepared from yhc8::kan and wild type strains growing under permissive conditions and from sec mutant cells after a 2-h shift to 38 °C. The extracts were electrophoresed through 7.5% SDS-polyacrylamide gel, blotted to nitrocellulose, and probed with anti-DPAP B serum. Bound antibodies were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). D, translocation of dipeptidyl-aminopeptidase B in Δyhc8-deleted strain. Yeast whole cell extracts were prepared from yhc8::kan and wild type strains growing under permissive conditions and from sec61-2 mutant cells after a shift to restrictive conditions. The extracts were electrophoresed through 12.5% SDS-polyacrylamide gel, blotted to nitrocellulose, and probed with anti-DPAP B serum. Bound antibodies were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).
bodies displayed a strong accumulation of fluorescent material in an intracellular organelle away from the nucleus (data not shown) which was probably the vacuole. This localization could result from a mistargeting due to the HA tag and/or be a consequence of its overexpression in conditions of GAL1 induction. The HA tag was exchanged for a protein A tag, and the induction conditions were changed. The cells were grown on 2% raffinose, a non-repressible carbon source (20). At time \( t = 0 \), the cells were transferred into a pre-warmed medium containing 2% galactose, and aliquots were fixed and decorated at 0, 30, and 60 min. Under these conditions, only few cells were decorated by the anti-protein A antibodies, and this proportion did not increase with the incubation time on galactose. Results in Fig. 4 show that the cells were primarily labeled at the periphery of the nucleus and the plasma membrane. This pattern is characteristic of the endoplasmic reticulum location. At 60 min, the cells appeared to be more heavily decorated, and the labeling around the nucleus appeared to be more diffuse. We concluded that Yhc8p was first directed to the endoplasmic reticulum membrane as suggested by the structural features of its sequence. Upon its accumulation in this compartment, the overproduced polypeptides were then transferred to the vacuolar compartment.

**DISCUSSION**

We have identified four coding sequences, YHC8, Hre556, Scyn1283, and UNP378, in the genome of *S. cerevisiae*, as homologues (43, 33.5, 34.5, and 32.5% amino acid sequence identity, respectively) of the Tsr1 gene of *Y. lipolytica*. Our study on Tsr1p suggested that it is localized in the ER membrane and is an important component of the SRP-dependent translocation pathway (15, 16). The proteins encoded by these Tsr1 homologues share high homology in both the amino-terminal and cytosolic domains; these two domains were demonstrated to be involved in the interaction of Tsr1p with BiP and with the SRP-ribosome complex, respectively (16). We called this new family of genes, Tsr1 family. Homology of the members of this family with Tsr1p and mutational test on YHC8 gene suggest that they may be involved in the SRP-dependent translocation pathway.

Here we focused on YHC8 because its putative product, Yhc8p, was most closely matched with Tsr1p. By using immunofluorescence experiments, we showed that Yhc8p is localized in the ER. The presence of an amino-terminal signal sequence and of a membrane-spanning domain suggested that Yhc8p, like its homologue Tsr1p, is a component of the ER membrane. We have demonstrated that deletion of one member of this family, YHC8 gene, although without effect on viability, induces large defects in the translocation of secretory soluble proteins, resulting in the accumulation of preinertase, pre-DPAP B, pre-pro-\( \alpha \)-factor. Only a slight defect was observed on the translocation of pre-DPAP B.

Previous studies have shown that mutations in sec61, sec62, and sec63 lead to a large accumulation of precursors of several secretory and soluble vacuolar proteins, such as \( \alpha \)-factor precursor, CPY, and acid phosphatase (5, 36) (see Fig. 3, A and C, 1st to 3rd lanes). However, these mutations have only marginal defects on the insertion of the integral membrane protein dipeptide-aminopeptidase B (DPAP B) (4) (see Figs. 3D, 1st to 3rd lanes). Other genetic screenings permitted identification of new mutants in the same genes that were defective in the insertion of integral membrane proteins (4). More recently, Pilon and colleagues (37) have characterized strains of *S. cerevisiae* expressing cold-sensitive alleles of SEC61 and show that these mutants exhibit a large cytoplasmic accumulation of co- and post-translationally translocated precursors. All together these data pointed to a model where Sec61p acts as the core of the translocon, controlling both the docking step onto the receptor site and insertion/translocation, whereas Sec63p and Sec62p were implicated specifically in the SRP-independent translocation pathway (1, 4, 11).

Our results are consistent with those obtained for the sec61, sec62, and sec63 mutants where the level of accumulation of precursors was dependent on the allele involved and the reporter protein used (4) (see Fig. 3A, 1st lane, and Fig. 3C, 1st lane). Only a small accumulation of ER forms was detected in the cases of inertase, CPY, and \( \alpha \)-factor compared with that observed in sec18-1 mutant. The sec18-1 mutant has been isolated as a thermosensitive mutant that exhibits a block in protein transport from the ER to the Golgi apparatus (38, 39), resulting from impaired targeting of the vesicles to an early Golgi compartment (40). The results obtained with Yhc8p suggest that Yhc8p controls primarily the translocation step and has only little effect on ER glycosylation. This could explain why the translocation defect and the accumulation of ER intermediates were more or less pronounced and dependent on the reporter protein used.

Our data show that the secretory defect in Yhc8p mutant is pleiotropic. Why is the null phenotype of the YHC8 gene not lethal? The fact that four homologues of the TSR1 gene have been identified in this yeast suggests that the products of these remaining three genes cooperate to allow partial suppression of the yhc8 null. Our results with the Yhc8p mutant are reminiscent of those with SEC71 and SEC72 mutants, which show pleiotropic defects in protein trafficking across the ER membrane but do not affect cell viability (10, 12). The Tsr1p gene product which was studied in more detail in *Y. lipolytica* was shown to interact with the SRP-ribosome complex on the cytoplasmic side and with BiP in the ER lumen in the predominant SRP-dependent translocation pathway in this yeast (16). If Yhc8p is involved in the same process, it is difficult to understand why the prepro-\( \alpha \)-factor and CPY, which were determined to be post-translationally translocated (41), are affected in the Yhc8p mutant cells. One explanation is that the loss of Yhc8p could induce a large decrease in the number of sites accessible for post-translational translocation. The fact that Tsr1p interacts with the SRP ribosome complex and deletion of YHC8 gene induces a large translocation defect suggests that Yhc8p may be an intermediary between the docking site on the SRP-receptor and the SEC61 complex, allowing it to play a general role in co- and post-translational translocation.

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