Identification of a novel member of yeast mitochondrial Hsp70-associated motor and chaperone proteins that facilitates protein translocation across the inner membrane

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Abstract Here, we report the identification of yeast 15-kD Tim15/Zim17, a new member of mitochondrial Hsp70 (mtHsp70)-associated motor and chaperone (MMC) proteins. The 15-kD MMC protein is a peripheral inner membrane protein with a zinc-finger motif. Depletion of the 15-kD protein led to impaired import of presequence-containing proteins into the matrix in vivo and in vitro. Overexpression of the 15-kD protein rescued the functional defects of mtHsp70 in *ssc1-3* cells, and a fusion protein containing the 15-kD protein physically interacts with purified mtHsp70. Tim15/Zim17 therefore cooperates with mtHsp70 to facilitate import of presequence-containing proteins into the matrix.

Keywords: Mitochondrial protein import; Hsp70; Yeast; Zinc-finger protein; DnaJ-like protein

1. Introduction

Most mitochondrial proteins are synthesized on the cytoplasmic ribosomes and imported into mitochondria [1–3]. Protein import into mitochondria requires translocators, the TOM (the translocase of the outer mitochondrial membrane) complexes in the outer membrane and the TIM (the translocase of the inner mitochondrial membrane) complexes in the inner membrane. Presequence-containing proteins and polytopic presequence-less proteins use the TIM23 or TIM22 complex, respectively, to move across or assemble into the inner membrane. The mitochondrial matrix also contains *m*itochondrial Hsp70 (mtHsp70)-associated *m*otor and *c*haperone (MMC) proteins that facilitate protein import into and/or protein folding in the mitochondrial matrix. These MMC proteins include mtHsp70 in the matrix and its partner proteins, Tim44, Tim14/Pam18 and Tim16/Pam16 in the inner membrane, and Yge1p/Mge1p and Mdj1p in the matrix [3,4].

Here, we searched for a new MMC protein, if any, that mediates protein import and/or protein folding in the matrix. For this purpose, we systematically analyzed the localization of yeast proteins that are indicated in the database to have essential but unknown functions [5,6]. We thus identified a novel essential 15-kD MMC protein encoded by *YNL310C*, which is involved in protein translocation through the TIM23 complex.

2. Materials and methods

2.1. Yeast strains and growth conditions

A haploid strain (Δtim15/pRS316-Tim15) whose chromosomal disruption of *YNL310C* was constructed by tetrad analysis of the diploid strain TIM15/Δtim15 harboring pRS316-Tim15 after sporulation, and the TIM15/Δtim15 strain by introducing a DNA fragment containing the *Candida glabrata HIS3* gene flanked by 40 base pairs of the sequences upstream and downstream of *YNL310C* into the wild-type (WT) diploid strain W303-AB. GAL-TIM15 was constructed by following the previously described procedure [7].

Yeast strains were grown in YPGal (1% yeast extract, 2% polypeptone, and 2% galactose), YPD (1% yeast extract, 2% polypeptone, and 2% glucose), and SCD (−Ura) (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, and 2% glucose without supplement of uracil).

2.2. In vitro protein import into mitochondria

The radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysates in the presence of ^{35}S-methionine. Import reactions were performed as described previously [7].

3. Results and discussion

3.1. A 15-kD protein encoded by YNL310C is an essential mitochondrial protein

The gene product of *YNL310C* is deposited as an essential protein in the *Saccharomyces cerevisiae* genome database (SGD). We found that the translation of *YNL310C* starts from the second ATG in the database (data not shown) and termed

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Abbreviations: TOM, the translocase of the outer mitochondrial membrane; TIM, the translocase of the inner mitochondrial membrane; mtHsp70, mitochondrial Hsp70; MMC, mitochondrial Hsp70-associated motor and chaperone; FOA, fluoroorotic acid; PK, proteinase K; DHFR, mouse dihydrofolate reductase; IMS, intermembrane space; mtHsp60, mitochondrial Hsp60; AAC, ADP/ATP carrier; GST, glutathione *S*-transferase

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its gene product as Tim15. A search of databases revealed that the gene product of *YNL310C* has homologs in a wide range of eukaryotic organisms (Fig. 1A). Similarity was observed throughout the sequence including the zinc-finger motif ($C^{75}XXC^{78}$ and $C^{100}XXC^{103}$).

We constructed a yeast haploid strain whose chromosomal *YNL310C* disruption was complemented by *YNL310C* on a *URA3* plasmid (Fig. 1B, *TIM15*). Cells losing the *URA3* plasmid can be selected on a 5′-fluoroorotic acid (5′-FOA) plate. We thus analyzed the abilities of $Tim15^{C75S}$ and $Tim15^{C100S}$ to complement the *YNL310C* disruption on the 5′-FOA plate. Cells with the gene for $Tim15^{C75S}$ (Fig. 1B, *tim15 C75S*) or $Tim15^{C100S}$ (Fig. 1B, *tim15 C100S*) did not grow on the 5′-FOA plate, suggesting that Cys75 and Cys100 of the zinc-finger motif are essential for the function of Tim15.

Since Tim15 is predicted to possess a mitochondrial targeting signal at the N-terminus, we analyzed its mitochondrial localization by proteinase K (PK) treatment of mitochondria and mitoplasts, where the outer membrane was ruptured, and found that Tim15 behaved like Mdj1p, a matrix protein partially associating with the inner membrane (Fig. 1C). Tim15 was, like Mdj1p and a peripheral inner membrane protein Tim44, extracted by alkaline treatment of mitochondria, but was not released to the supernatant by sonication followed by ultracentrifugation (Fig. 1D). These results indicate that Tim15 is a peripheral membrane protein of the inner membrane facing the matrix.

The radiolabeled Tim15 precursor was synthesized in vitro and incubated with isolated mitochondria. Tim15 was imported into mitochondria in a membrane potential ($\Delta\Psi$)-dependent manner (Fig. 1E). The presequence of Tim15 is most likely cleaved at the predicted cleavage site past residue 42 to yield a 14.9-kDa mature form, since the size of the mature form was close to that of truncated Tim15 starting from residue 43 on the SDS–PAGE gel (Fig. 1E, lane 1).

During preparation of the manuscript on this study, identification of the gene product of *YNL310C* was reported on-line, which was termed Zim17 [8]. Because the name of Zim17 does not reflect its molecular mass (14.9 kD), we tentatively call it Tim15 according to the original rule of the agreed nomenclature of the proteins involved in mitochondrial protein transport [9].

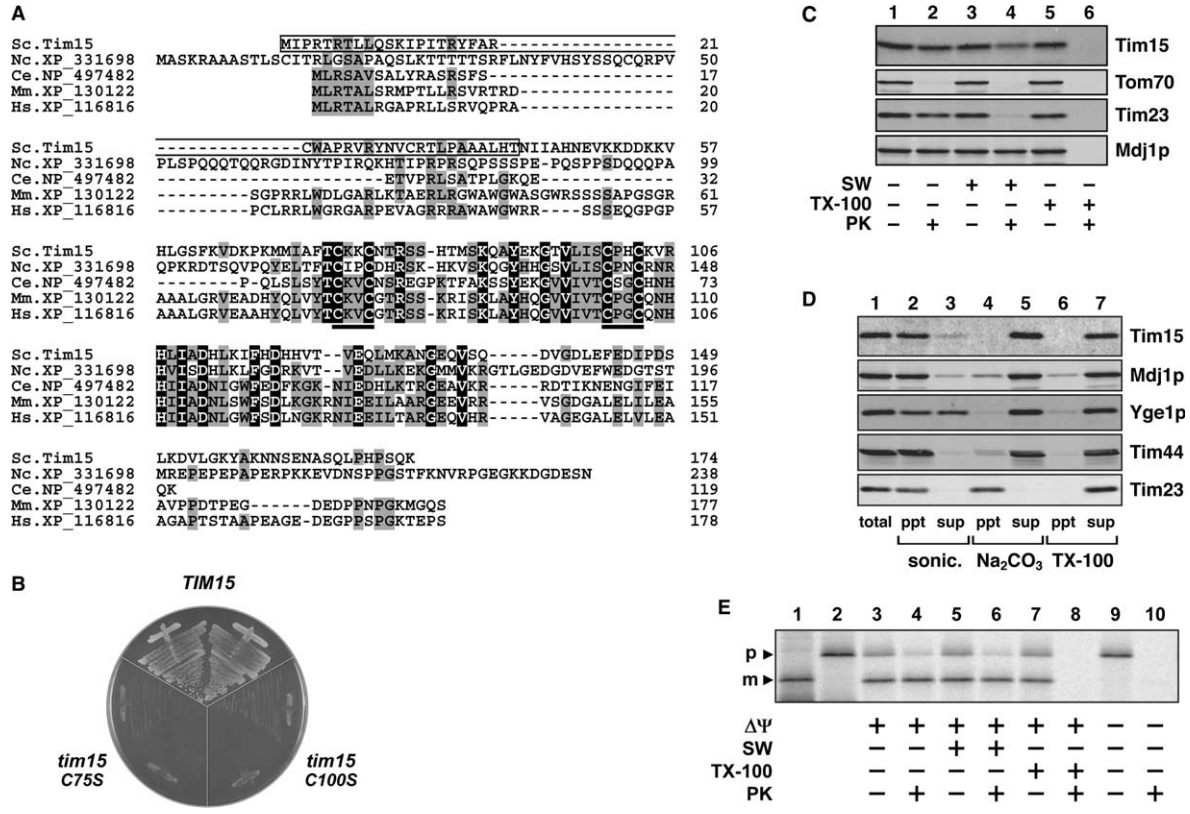

Fig. 1. Tim15/Zim17. (A) Predicted amino-acid sequence of Tim15/Zim17. Sc, *S. cerevisiae*; Nc, *Neurospora crassa*; Ce, *Caenorhabditis elegans*; Mm, *Mus musculus* ; Hs, *Homo sapiens*. Identical residues are shown in black and similar residues in gray. The presequence is boxed, and conserved zinc-finger motifs are underlined. (B) A *TRP1* single-copy plasmid harboring the wild-type *TIM15* gene (*TIM15*), that with a mutation for C75S (*tim15 C75S*) or for C100S (*tim15 C100S*) was introduced into the same haploid strain (Δtim15/pRS316-Tim15), whose chromosomal disruption of *YNL310C* was complemented with a *URA3* plasmid of *YNL310C*. Yeast strains dependent on mutated *TIM15* on the *TRP1* plasmid were selected on 5′-FOA plates and their growth was compared at 30 °C. (C) Mitochondria and mitoplasts generated by osmotic swelling (SW) were treated with 50 μg/ml PK for 30 min at 25 °C in the presence or absence of 0.1% Triton X-100 (TX-100). Proteins were detected by immunoblotting with antibodies against indicated proteins. (D) Mitochondria were treated with sonication (sonic.), 0.1 M Na_2CO_3 at pH 10.8 (Na_2CO_3), or 1% Triton X-100 with 500 mM NaCl for 30 min on ice (TX-100). Pellets (ppt) and supernatants (sup) were then separated by centrifugation (100 000 × *g* for 30 min). Proteins were detected by immunoblotting. (E) In vitro import of the Tim15 precursor into isolated yeast mitochondria for 20 min at 23 °C with or without $\Delta\Psi$. The mitochondria were then subjected to osmotic swelling (SW) or treatment with 0.1% Triton X-100 (TX-100) and further treated with or without 100 μg/ml PK for 20 min on ice. The mitochondria or mitoplasts were reisolated by centrifugation, and proteins were analyzed by SDS–PAGE and radioimaging. Lane 1, a putative mature form of Tim15 starting from residue 43; lane 2, the Tim15 precursor (10% control).