Protein import into yeast mitochondria

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Identification of the Tim17 orthologues of *Kluyveromyces lactis* and *Schizosaccharomyces pombe* and complementation analysis of a *Saccharomyces cerevisiae* tim17 null strain

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Chapter 3
Identification of the Tim17 orthologues of K. lactis and S. pombe

Abstract

We have cloned the gene of Kluyveromyces lactis and the cDNA of Schizosaccharomyces pombe encoding the mitochondrial inner membrane protein translocase Tim17 by means of colony hybridization using the TIM17 gene of Saccharomyces cerevisiae as a hybridization probe. DNA sequence analysis revealed the open reading frames coding for the protein sequences of K. lactis and S. pombe Tim17 indicating 90% and 66% identity to Tim17 of S. cerevisiae, respectively. These are the first identified Tim17 orthologues able to functionally complement an S. cerevisiae tim17 null mutant.

Introduction

Most mitochondrial proteins are encoded by the nucleus and synthesized with an N-terminal targeting signal that directs them to the mitochondria. Import of these preproteins into the mitochondria is mediated by two multisubunit translocases, the Tom complex in the outer membrane and the Tim complex in the inner membrane. The Tom complex consists of at least nine subunits that mediate the recognition of precursor proteins followed by translocation through the import channel of the outer membrane [19, 50, 67, 72, 83, 103, 175, 196]. Subsequent translocation of precursors across or into the inner membrane is mediated by two distinct systems of Tim proteins. One Tim system consists of the transmembrane proteins Tim17 and Tim23, the matrix localized peripheral membrane protein Tim44 and the matrix chaperone Hsp70 (mtHsp70). Tim23 and Tim17 are proposed to constitute the translocation channel and to cooperate with the ATP-dependent Tim44-mtHsp70 translocation motor to translocate preproteins across the inner membrane [45, 54, 127, 128]. While the Tim17-Tim23 complex probably mediates import of all matrix proteins and a few intermembrane space proteins, another recently discovered system is responsible for directing a number of polytopic membrane proteins such as members of the mitochondrial carrier family to the inner membrane. Seven components of this second Tim translocator complex have now been identified [101, 106-108, 186]. The exact molecular mechanism of protein translocation is for both systems still unknown. In the case of the Tim17-Tim23 complex we do not know which protein domains or specific amino acids are important for the formation of the aqueous channel and the selective passage of preproteins while preventing leakage of protons that would otherwise disturb mitochondrial function.

Here we describe the identification of the TIM17 orthologues from two other yeast species, Kluyveromyces lactis and Schizosaccharomyces pombe. The K. lactis TIM17 gene and the S. pombe TIM17 cDNA were identified by colony hybridization with a probe consisting of the complete open reading frame of the S. cerevisiae TIM17 gene. The TIM17 orthologues were
subjected to DNA sequence analysis and their deduced amino acid sequences were compared in a multiple sequence alignment with five other Tim17 orthologues, to analyze the evolutionary conservation of amino acid residues important for the function of Tim17. Functional complementation analysis showed that the Tim17 orthologues of *K. lactis* and *S. pombe* are the first orthologues that can functionally replace Tim17 of *S. cerevisiae*.

**Materials and Methods**

**Strains and media**

*Escherichia coli* strain JF 1754 (lac gal metB leuB hisB hsdR) was used for DNA manipulations. *E. coli* transformants were grown in YT medium (1.6% (w/v) bactotryptone, 1% (w/v) yeast extract and 0.5% (w/v) NaCl) containing 200 μg/ml ampicillin. *S. cerevisiae* strain MB26 (ade2-101 trp1-289 ura3-52 his3-Δ200 tim17::LYS2 leu2-Δ1 + YCplac33::TIM17) [128] was used for performing the complementation analysis. Transformation of yeast was performed according to Klebe *et al.* [104]. Double transformants were selected on minimal media containing 0.67% (w/v) yeast nitrogen base (Difco), 2% (w/v) glucose, 2% (w/v) agar supplemented with adenine, tryptophan and histidine (20 μg/ml). Double transformants were grown overnight in YPD medium (2% (w/v) glucose, 1% (w/v) yeast extract, 2% (w/v) bactopeptone) and plated on minimal media containing 1 g/l 5-fluoroorotic acid (5-FOA) [14], 0.67% (w/v) yeast nitrogen base (Difco), 2% (w/v) glucose, 2% (w/v) agar supplemented with adenine, tryptophan, histidine and uracil (20 μg/ml).

**DNA manipulations**

The genomic DNA library of *K. lactis* (gift of Joris Heus) contained fragments ranging from 0-10 kb ligated into the vector YRp14/KARS2 [79]. Transformation of *E. coli* with the library yielded about 7000 transformants. After transfer to nitrocellulose filters, one positive colony was identified by colony hybridization with an *S. cerevisiae* TIM17 probe. A 7 kb SphI insert containing the *K. lactis* TIM17 gene was subcloned into the SphI site of YCplac111 [63]. Further subcloning and Southern blot analysis revealed that a 1.6 kb EcoRI and a 2.7 kb HindIII fragment contained the *K. lactis* TIM17 gene. The 1.6 kb EcoRI fragment was cloned into the EcoRI site of YCplac111 and the 2.7 kb HindIII fragment was cloned into the HindIII site of YCplac111. Both constructs were used for genetic complementation analysis of the *S. cerevisiae* tim17 null strain MB26 and only the first construct was used for determination of the DNA sequence.

In the cDNA library from *S. pombe* (gift of John Fikes) cDNA fragments were cloned behind the constitutive *ADH1* promoter of the vector pDB20 [57]. The library was transformed into *E. coli*, yielding about 8000 transformants which were then transferred to nitrocellulose filters. One positive colony was identified by colony hybridization with an *S. cerevisiae* TIM17 probe. A blunted 950 bp HindIII fragment containing the complete *S. pombe* cDNA insert was subcloned into the Smal site of YCplac111::ADH1p [128]. Expression of the *S. pombe* TIM17 gene is thereby under control of the *ADH1* promoter. This construct was used for DNA sequence analysis and complementation analysis of the *S. cerevisiae* tim17 deletion mutant MB26.

**Colony hybridization**

*E. coli* transformants were transferred to Hybond nitrocellulose filters which were then incubated in prehybridization buffer (6x SSC, 5x Denhardt’s, 100 μg/ml salmon sperm DNA, 0.5% SDS) for 3 h. at 65°C. Blots were hybridized overnight at 65°C with an *S. cerevisiae* TIM17 probe and then washed with 3x SSC/0.1% SDS, 2x SSC/0.1% SDS and 1x SSC/0.1% SDS at 65°C for 30 min. each, followed by autoradiography on Hyperfilm (Amersham). The TIM17 probe consisted of a DNA fragment liberated from YCplac111::tim17-2. This vector, which contains a mutant TIM17 gene with an EcoRI restriction
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site introduced 3 bp downstream of the initiator ATG, was digested with *EcoRI* and *HindIII* (*HindIII restriction site is located 5 bp upstream of the stop codon*). This yielded a 473 bp *EcoRI-HindIII* fragment which lacked the first 2 bp and the last 3 bp of the *TIM17* open reading frame. This fragment was radiolabeled by nick-translation in the presence of α-32P-dATP according to standard procedures [163].

**DNA sequence analysis**

YCplac11 containing the *K. lactis TIM17* gene was subjected to DNA sequence analysis according to Sanger *et al.* [164] using M13 primers and *K. lactis TIM17* specific primers. YCplac11::ADH1p containing the *S. pombe TIM17* cDNA was sequenced using M13 primers, an *ADH1* promoter specific primer and *S. pombe TIM17* specific primers. The *K. lactis TIM17* gene and *S. pombe TIM17* cDNA were sequenced on both strands.

**Miscellaneous**

Restriction enzymes were purchased from Biolabs, Gibco or Boehringer. Radiolabeled α-32P-dATP, Hybond nitrocellulose filters and Hyperfilms were obtained from Amersham. A GCG sequence analysis package was made available by the CAOS/CAMM centre, Nijmegen.

**Results**

**Cloning of the TIM17 gene of *Kluyveromyces lactis* and the TIM17 cDNA of *Schizosaccharomyces pombe***

*E. coli* was transformed with the *K. lactis* DNA library [79] and the *S. pombe* cDNA library [57], yielding about 7000 and 8000 transformants, respectively. These transformants were transferred to Hybond nitrocellulose filters and subjected to colony hybridization with a probe consisting of the entire *S. cerevisiae TIM17* gene (see Materials and Methods). Autoradiography revealed one positive colony amongst the transformants with *K. lactis* DNA and one positive colony amongst the colonies transformed with DNA of the *S. pombe* library.

Restriction analysis of the plasmid DNA isolated from the positive colony harbouring *K. lactis* DNA revealed that the clone contained an insert of approximately 8.5 kb. Southern blot analysis with the *S. cerevisiae TIM17* probe indicated that at least part of the *K. lactis TIM17* gene was located on a 1.6 kb *EcoRI* and a 2.7 kb *HindIII* fragment. Both fragments were cloned into the single copy shuttle vector YCplac11 [63] and then used for functional analysis in *S. cerevisiae* and the vector with the 1.6 kb *EcoRI* fragment was used for DNA sequence analysis. *S. pombe TIM17* could be isolated as a 950 bp *HindIII* insert from the positive *E. coli* transformant. This *HindIII* fragment was cloned in both orientations in YCplac11::ADH1p [128] behind the *ADH1* promoter and this vector was used for functional analysis in *S. cerevisiae* and determination of the DNA sequence.
Figure 1. DNA and cDNA sequence and deduced amino acid sequences of TIM17 of K. lactis (Figure 1a) and S. pombe (Figure 1b), respectively. The stop codon is indicated (*). The amino acid sequence is given in the one-letter code. The four predicted transmembrane domains are underlined.
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Figure 2. Multiple sequence alignment of the protein sequences of known Timl7 orthologues. Sequences were aligned using the GCG PileUp program (gap weight 8.00; gap length weight 3) [56]. Conserved amino acids are indicated by a black box, similar amino acids by a grey box. Gaps are indicated (.). k.l. = *K. lactis* Tim17 [this study]; s.c. = *S. cerevisiae* Tim17 [128] (acc. nr. X77796); s.p. = *S. pombe* Tim17 [this study]; h.s. = *Homo sapiens* Tim17 [20] (acc. nr. Z46191); r.n. = rat Tim17 [92] (acc. nr. O35092); m.m. = *Mus musculus* Tim17 (acc. nr. AAD19595); d.m. = *Drosophila melanogaster* Tim17 [20] (acc. nr. L35645); c.e. = *Caenorhabditis elegans* Tim17 [20] (acc. nrs. D74639, D75891, D74501); a.t. = *Arabidopsis thaliana* Tim17 [20] (acc. nr. T45278).
DNA sequence analysis of the *TIM17* orthologues of *K. lactis* and *S. pombe*

DNA sequence analysis revealed that the *K. lactis* and *S. pombe* DNA fragments both contained the sequence of a gene that could be translated into a protein with high homology to Tim17 of *S. cerevisiae*, strongly suggesting that we had cloned the *TIM17* gene of both *K. lactis* and *S. pombe* (Figure 1).

The *K. lactis* *TIM17* gene encompasses an open reading frame of 465 bp encoding a protein of 155 amino acids with a predicted molecular weight of 16.3 kDa. The *S. pombe* cDNA comprises an open reading frame of 495 bp encoding a protein of 165 amino acids with a calculated molecular weight of 16.9 kDa. The cDNA sequence analysis also revealed a G to C change at position 356, resulting in an amino acid substitution at position 119 (cysteine to serine) in comparison to the DNA sequence deposited in the GenBank Database (accession numbers Z95395 and P87130). Since in *S. pombe* the *TIM17* gene is encoded by two exons, splicing will occur. The predicted splice donor and acceptor sequences deposited in the GenBank Database was confirmed by our DNA sequence analysis. Multiple sequence alignment of the amino acid sequences of the Tim17 proteins of *S. cerevisiae*, *K. lactis* and *S. pombe* using the GCG PileUp Program (Figure 2) revealed an identity of 90% between *S. cerevisiae* and *K. lactis*, and of 66% between *S. cerevisiae* and *S. pombe*. Hydrophilicity plots of the *K. lactis* and *S. pombe* proteins predict the presence of the same four hydrophobic domains such as postulated for *S. cerevisiae* Tim17 (data not shown) [45, 114]. These features all together suggest that we have cloned the gene and cDNA encoding Tim17 of *K. lactis* and *S. pombe*, respectively.

**Functional complementation analysis**

Alignment of all currently known Tim17 orthologues shows that the protein is highly conserved in evolution (Figure 2 and [20]). Despite this, however, Tim17 orthologues of *Drosophila melanogaster* and *Homo sapiens* are unable to substitute Tim17 of *S. cerevisiae* (M. Meijer and A.C. Maarse, unpublished results).

To determine whether the Tim17 orthologues of *K. lactis* and *S. pombe* are functional in an *S. cerevisiae* genetic background, a complementation analysis was performed in a yeast strain lacking the endogenous *TIM17* gene, MB26. This strain is not able to grow on media with any carbon source, but remains viable due to the presence of the single copy shuttle vector YCplac33 (*URA3* marker) containing the wild type *TIM17* gene of *S. cerevisiae* [128]. The single copy shuttle vector YCplac111 or YCplac111::ADH1p containing either the *K. lactis* *TIM17* gene or *S. pombe* *TIM17* cDNA respectively, were then transformed to MB26 and double transformants were selected on minimal media, subsequently grown overnight in YPD and finally, samples were plated on minimal medium containing 5-fluoroorotic acid (5-FOA). Cells that synthesize the *URA3* gene product orotidine-5'-phosphate decarboxylase [14] convert the 5-FOA into the toxic 5-fluoro-uracil and will die. In the case that *K. lactis* and *S. pombe* *TIM17* are functional in
The double transformants can lose the plasmid harbouring the *S. cerevisiae* TIM17 gene and the *URA3* marker under non-selective growth conditions, allowing subsequent growth of the cured strain MB26 on 5-FOA plates. As controls for this plasmid shuffling assay, MB26 was also transformed with the vector YCplac111 and the plasmid YCplac111::ScTIM17. MB26 transformed with the plasmid containing the 1.6 kb *EcoRI* or the 2.7 kb *HindIII* fragment covering the *K. lactis* TIM17 gene were able to grow on 5-FOA plates and growth was comparable to cells harbouring *S. cerevisiae* TIM17 (Figure 3, complementation of 2.7 kb *HindIII* fragment is not shown). This indicates that a functional *K. lactis* Tim17 was expressed from both plasmids. Only transformants containing the plasmid with the *S. pombe* TIM17 cDNA fragment in the correct orientation were able to grow on 5-FOA plates, whereas cells containing this cDNA fragment in the opposite orientation did not grow (Figure 3, complementation of cDNA fragment in opposite orientation not shown). This indicates that the *S. pombe* Tim17 protein was only expressed when the cDNA was cloned in the correct orientation behind the *ADH1* promoter. Cells transformed with YCplac111 plasmid DNA did not show any detectable growth as expected. These results indicate that the Tim17 orthologues of *K. lactis* and *S. pombe* can complement an *S. cerevisiae tim17* null mutant. The Tim17 orthologues of *K. lactis* and *S. pombe* are the first Tim17 orthologues identified that are able to functionally substitute for Tim17 of *S. cerevisiae*.

**Figure 3.** Functional complementation analysis of the Tim17 orthologues of *K. lactis* and *S. pombe* in an *S. cerevisiae tim17* deletion strain. Complementation analysis of *tim17* null strain MB26 transformed with either YCplac111, YCplac111::ScTIM17, YCplac111::KlTIM17 or YCplac111::SpTIM17. Double transformants were selected on minimal media, then grown overnight in rich medium (YPD) and subsequently plated on 5-FOA containing minimal medium. Growth at 23°C was monitored for several days and compared with growth of MB26 transformed with YCplac111::ScTIM17 (normal growth) or the vector YCplac111 (no growth).
Chapter 3

Discussion

We have cloned the nuclear gene and a cDNA encoding the Tim17 orthologues of *K. lactis* and *S. pombe*, respectively. DNA sequence analysis revealed that the *TIM17* gene of *K. lactis* encodes a protein with 90% identity to Tim17 of *S. cerevisiae*. The *TIM17* cDNA of *S. pombe* encodes a protein that is 66% identical to the *S. cerevisiae* orthologue. Alignment of the Tim17 protein sequences of these three yeast species indicates that they are very similar (Figure 2). The homology of the proteins is also apparent from the presence of four hydrophobic domains that may function as inner membrane anchors, and from the absence of a cleavable N-terminal presequence [45, 128]. Their homology is also indicated by a complementation analysis, which shows that Tim17 orthologues of *K. lactis* and *S. pombe* can complement an *S. cerevisiae* timl7 null mutant. The Tim17 proteins of *K. lactis* and *S. pombe* are the first Tim17 orthologues identified that can function in *S. cerevisiae*.

Previously, Tim17 encoding sequences of six other organisms have been identified and alignment revealed a similarity ranging from 70 to 82% [20, 92]. The orthologues of *Drosophila melanogaster* and *Homo sapiens* were cloned, expressed *in vitro* and successfully imported into mitochondria isolated from *S. cerevisiae*. Even though these species are at a large evolutionary distance from each other, the imported proteins appeared to assemble in their correct topology in the inner membrane. However, they were not able to complement the *S. cerevisiae* strain MB26 (M. Meijer and A.C. Maarse, unpublished results). These results imply that the mitochondrial targeting signal of Tim17 is conserved in evolution and that it can be recognized by the protein import system of *S. cerevisiae*. Lack of functional complementation is obviously not due to inefficient import or incorrect assembly of Tim17 into the inner membrane translocase complex, but may possibly be attributed to an evolutionary divergence of amino acids that are important for function of Tim17 in the Tim complex. The multiple sequence alignment in Figure 2 shows that the conservation of a number of amino acid residues is restricted to the Tim17 orthologues of the three yeast species. These amino acids may be important for the function of Tim17 and absence of these residues may result in lack of functionality of Tim17 in *S. cerevisiae*.

The information for the import of Tim17 into mitochondria is present within the mature part of the protein sequence [128]. Recently, it was proposed by Káldi *et al.* [97] that this targeting signal (IS17, internal signal 17) is located in the hydrophobic C-terminal region of the protein (between the third and fourth predicted transmembrane segments, encompassing amino acids 103 to 112). A fusion of IS17 and a passenger protein sequence results in a chimeric protein that can be imported into mitochondria [97]. Alignment of the Tim17 orthologues (Figure 2) shows a similarity of this region for the three yeast species, which does not extend to Tim17 of other organisms. Since the human and *D. melanogaster* orthologues can be imported into mitochondria, this may imply that either for these orthologues evolutionary divergence of IS17...
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has no effect on the import efficiency, or that IS17 may function in cooperation with other, yet unidentified, species-specific elements involved in import. It may also indicate that the primary sequence is not the only determinant of a mitochondrial targeting signal and that other factors, such as the ability to form an amphipathic α-helix, are involved. Another possibility may be that not IS17, but another domain in Tim17 is necessary for import into mitochondria [195]. Controversial results have been obtained for the location of the mitochondrial targeting sequence in Tim23, a protein with high homology to Tim17 in protein sequence and structural features. Davis *et al.* [41] showed that the Tim23 targeting signal resides in the first and fourth hydrophobic transmembrane segment of the protein, whereas Káldi *et al.* [97] showed that the internal targeting signal of Tim23 (IS23, internal signal 23) was located in a region encompassing amino acids 186 to 197. In view of this controversy and considering the high protein sequence and structural similarity of Tim17 and Tim23, it may be speculated that additional sequences of Tim17 may function as a targeting signal. Mutagenesis studies in *S. cerevisiae* showed that an insertion of just two amino acids in the region encompassing the proposed targeting signal IS17 (at position 111) gives rise to a mutant Tim17 protein that is no longer functional (J. Blom, unpublished results). Whether this dysfunctionality is due to a mutation in the targeting signal resulting in inefficient import, or caused by a mutation in a domain important for the function of Tim17, remains to be investigated. Although the region near amino acid 111 is not conserved in the human and *D. melanogaster* Tim17 orthologues, they are imported into mitochondria. It is therefore tempting to speculate that lack of complementation of the *S. cerevisiae* Tim17 position 111 mutant is the result of a mutation in a functional domain of the protein and not due to inefficient import.

Alignment shows that sequence conservation is less pronounced in the C-terminal region of all Tim17 orthologues, suggesting that this region may be less important for the function of the protein. The finding that regions within a protein display a pronounced sequence conservation suggests that these regions have a functional significance and are interesting for further analysis by mutagenesis. Since the multiple sequence alignment of the Tim17 orthologues of various species (Figure 2) reveals a high degree of homology that is extended throughout the whole protein, it will be difficult to select amino acids that can be subjected to mutagenesis. Nevertheless, site-directed mutagenesis can offer a good opportunity to further analyze the function of Tim17.

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