A conserved proline residue is present in the transmembrane-spanning domain of Tom7 and other tail-anchored protein subunits of the TOM translocase

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Abstract The TOM translocase consists of several integral membrane proteins organised around the channel forming protein Tom40. Here we show that one of these protein subunits, Tom7, is a tail-anchored protein. The carboxy-terminal 33 amino acids of Tom7 contain the information for targeting the protein to the mitochondrial outer membrane, and a conserved proline residue within the transmembrane segment is required for efficient targeting of Tom7 to the outer membrane. An equivalent proline residue is important in targeting each of the other three tail-anchored proteins that associate with Tom40 to form the core of the TOM translocase.

Key words: Mitochondria; Membrane proteins; Protein targeting

1. Introduction

Most mitochondrial precursor proteins are synthesised on ribosomes in the cytosol, and are recognised and translocated across the mitochondrial outer membrane by the TOM translocase. In yeast, the core of the Tom translocase consists of five integral membrane proteins: Tom40, Tom5, Tom6, Tom7 and Tom22 [1,2]. Tom40 represents the protein translocation channel, and might be a β-barrel protein analogous to bacterial porins [3–5]. Standard hydropathy analyses predict a single, α-helical transmembrane segment towards the carboxy-terminus of Tom5, Tom6 and Tom22, and each has been experimentally verified to have an amino-terminal domain displayed in the cytosol and a shorter *trans* domain in the intermembrane space [6–8]. By this criteria, Tom5, Tom6 and Tom22 are tail-anchored proteins [9].

The last subunit of the core translocase, Tom7, is defined as an integral membrane protein based on biochemical criteria: alkali extraction of mitochondrial membranes does not remove Tom7 from the outer membrane [10,11]. Standard hydropathy algorithms do not predict any α-helical membrane-spanning segment from the primary structure of Tom7. However, we have recently developed parameters that allowed the prediction of a near complete list of tail-anchored proteins from genome data of *Saccharomyces cerevisiae*. This algorithm predicts Tom7 has a membrane-spanning segment that could traverse the mitochondrial outer membrane (Beilharz et al., in preparation).

Here we define the topology of Tom7 in the mitochondrial outer membrane, and demonstrate that like other tail-anchored proteins the membrane-spanning segment is required for targeting of Tom7 to mitochondria. This region of the protein has been well conserved through evolution and contains a single proline residue: replacement of this proline residue diminishes targeting to mitochondria and leaves Tom7 susceptible to proteolytic degradation in wild-type cells. All four of the subunits that interact with Tom40 to form the core translocase are tail-anchored proteins, and each has a conserved proline residue within the transmembrane segment that might facilitate interaction with the proposed β-barrel channel and the overall function of the core TOM translocase.

2. Materials and methods

2.1. Expression constructs and yeast strains

The 180 bp DNA fragment corresponding to the Tom7 open reading frame was amplified from genomic DNA using the polymerase chain reaction (PCR), and cloned into either the *Bam*HI site ($_N$Tom7$_C$–green fluorescent protein (GFP)) or *Bgl*II site (GFP–$_N$Tom7$_C$) of p416 MET25HDEL [15]. As a result, fusions are expressed under the control of the *MET25* promoter. The GFP–Δ6-28Tom7 and each of the proline replacement mutants Tom7(ΔP), Tom5(ΔP), Tom6(ΔP) and Tom22(ΔP) mutants were constructed by PCR, and each plasmid checked by DNA sequencing.

For fluorescence microscopy, yeast cells were visualised directly or after staining with Mitotracker (Mitotracker Red CM-H2X Ros) according to the standard protocol from Molecular Probes. All fluorescence images were captured using a Bio-Rad MRC1024 Confocal Scanning Laser microscope mounted on a Zeiss Axioscope. Where indicated, up to five serial scans were stacked into composite images.

2.2. Import assays

Translation and import of ^{35}S-labelled porin were as described previously [12], and mitochondria were subsequently separated from non-inserted and aggregated porin by sucrose density gradient centrifugation. The assay was based on a method described previously [13], but ^{35}S-labelled porin was presented to mitochondria isolated from the strains indicated and after incubation for 8 min at 25°C in import buffer (0.6 M sorbitol, 50 mM HEPES pH 7.4, 2 mM Kpi pH 7.4, 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol), mitochondria were extracted with 100 mM Na_2CO_3. The mitochondrial extracts were mixed with 2 M sucrose made up in 100 mM Na_2CO_3 to a final sucrose concentration of 1.6 M. Samples were transferred to an SW60 tube and overlaid with 1 ml of 1.3 M sucrose made up in water, followed by 1 ml of water (0 M sucrose). The samples were centrifuged at 28 000 rpm for 16 h. The mitochondrial

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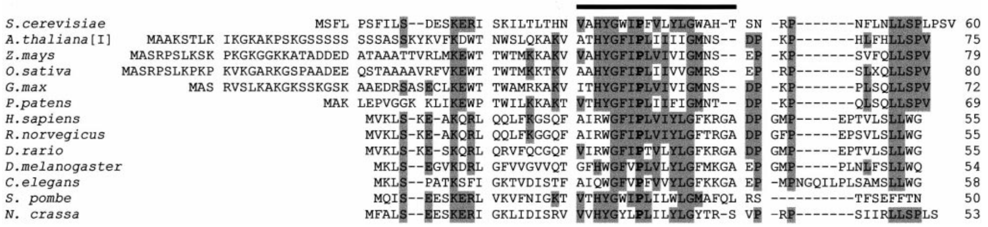

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S.cerevisiae                                       MSFL PSFILS--DESKERI SKILTLTHN VAHYGWIPFVLYLGWAH-T SN -RP-------NFLNLLSPLPSV 60
A.thaliana[I]     MAAKSTLK IKGKAKPSKGSSSSS SSSASSKYKVFKDWT NWSLQKAKV ATHYGFIPLIIIIGMNS-- DP -KP-------HLFHLLSPV    75
Z.mays          MASRPSLKSK PKGKGGKKATADDED ATAAATTVRLMKEWT TWTMKKAKV VAHYGFIPLVIVIGMNS-- EP -KP-------SVFQLLSPV    79
O.sativa       MASRPSLKPKP KVKGARKGSPAADEE QSTAAAAVRFVKEWT TWTMKKTKV AAHYGFIPLIIVVGMRS-- EP -RP-------SLXQLLSPV    80
G.max                  MAS RVSLKAKGKSSKGSK AAEDRSASECLKEWT TWAMRKAKV ITHYGFIPLVIVIGMNS-- DP -KP-------PLSQLLSPV    72
P.patens                                    MAK LEPVGGK KLIKEWP TWILKKAKT VTHYGFIPLIIFIGMNT-- DP -KP-------QLSQLLSPV    69
H.sapiens                                        MVKLS-KE-AKQRL QQLFKGSQF AIRWGFIPLVIYLGFKRGA DP GMP-----EPTVLSLLWG     55
R.norvegicus                                     MVKLS-KE-AKQRL QQLFKGGQF AIRWGFIPLVIYLGFTRGA DP GFP-----EPSVLSLLWG     55
D.rario                                          MVKLS-KE-SKQRL QRVFQCGQF VIRWGFIPTVLYLGFKRGA DP GMP-----EPTVLSLLWG     55
D.melanogaster                                    MKLS--EGVKDRL GFVVGVVQT GFHWGFVPLVLYLGFMKGA EP GMP-----PLNLFSLLWQ     54
C.elegans                                         MKLS--PATKSFI GKTVDISTF AIQWGFVPFVVYLGFKKGA EP -MPNGQILPLSAMSLLWG     58
S. pombe                                          MQIS--EESKERL VKVFNIGKT VTHYGWIPLILWLGMAFQL RS ----------TFSEFFTN     50
N. crassa                                        MFALS--EESKERI GKLIDISRV VVHYGYLPLILYLGYTR-S VP -RP-------SIIRLLSPLS   53
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Fig. 1. The primary structure of the predicted transmembrane domain of Tom7 is highly conserved throughout evolution. Sequence alignment of Tom7 related sequences from the indicated species of plants, vertebrates, invertebrates and fungi. Iterative BLAST analysis was used to identify the sequences and a ClustalW alignment is shown. Identical residues are shaded and the black line denotes the transmembrane segment predicted for Tom7 from *S. cerevisiae*.

membranes were harvested from the 1.3 M, 0 M sucrose interface for SDS–PAGE and phosphorimage analysis.

2.3. Miscellaneous

Published procedures were used for isolation of mitochondria and trypsin shaving, SDS–PAGE, BN–PAGE and immunoblot analysis [12,14].

3. Results

3.1. Prediction of a transmembrane segment in Tom7

Sequence analysis of all open reading frames in the genome of *S. cerevisiae* predicts 55 tail-anchored proteins, including Tom7 (Beilharz et al., in preparation). The membrane-spanning segment predicted in Tom7 extends from residue 27 to residue 45. This region of the protein has been highly conserved through evolution (Fig. 1).

3.2. Tom7 is tail-anchored in the mitochondrial outer membrane

In order to test the predicted transmembrane topology of Tom7, we constructed a set of GFP fusion proteins (Fig. 2A) and expressed these in yeast. Fusion of GFP to the amino-terminus of Tom7 (GFP–$_N$Tom7$_C$) resulted in a protein that was targeted to the mitochondria (Fig. 2B), as evidenced by the coincident staining with the specific dye Mitotracker (data not shown). When GFP was fused to the carboxy-terminus of Tom7 ($_N$Tom7$_C$–GFP) only very weak fluorescence was noted, and only in few cells, but where present the fluorescence appeared in mitochondria (data not shown). The GFP–$_N$Tom7$_C$ fusion protein is functional. Mitochondria isolated from Δ*tom7* cells have a reduced capacity to insert porin into the outer membrane [10], however expression of GFP–$_N$Tom7$_C$ complements the ability of the mutant cells to insert porin (Fig. 2C).

Mitochondria were isolated from cells expressing either GFP–$_N$Tom7$_C$ or $_N$Tom7$_C$–GFP and subjected to limited proteolysis to determine the topology of Tom7. Immunoblot analysis of mitochondria carrying the protein GFP–$_N$Tom7$_C$ revealed that trypsin destroyed the GFP epitope (Fig. 2D, '+'). The protease sensitive cytochrome b_2 is within the intermembrane space and protected from trypsin by the outer membrane ('+'), but in hypotonic buffer the outer membrane ruptures and trypsin can access the intermembrane space ('+M'). In mitochondria carrying the protein $_N$Tom7$_C$–GFP, the GFP epitope is largely within the intermembrane space and not completely degraded by trypsin until after the outer membrane is disrupted by hypotonic shock (Fig. 2E, '+M'). The outer membrane of these mitochondria tends to be fragile, as evidenced by the partial degradation of the cytochrome b_2 control even in isotonic buffer ('+'). We conclude that Tom7 is anchored in the mitochondrial outer membrane with a N$_{out}$–C$_{in}$ topology.

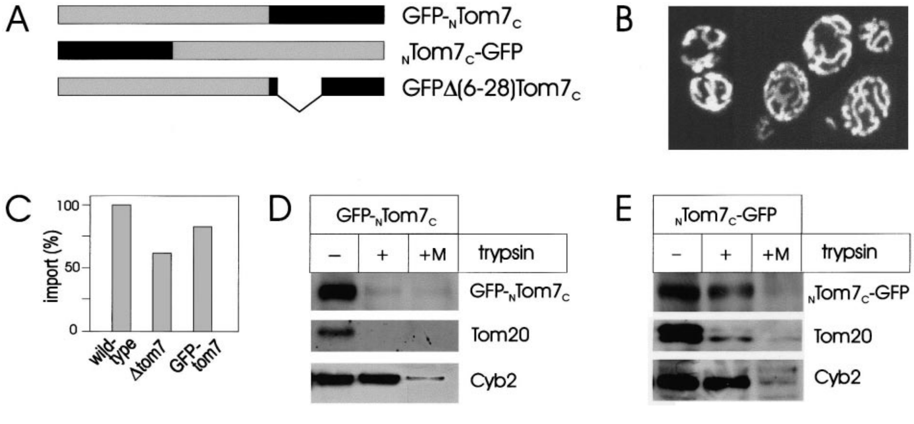

Fig. 2. Tom7 is tail-anchored in the mitochondrial outer membrane. A: Schematic representation of the fusion proteins used in this study. B: Yeast cells were transformed with a plasmid encoding the GFP–$_N$Tom7$_C$ fusion protein and living cells observed by fluorescence microscopy. A composite image of five stacked layers is shown to highlight the tubular morphology characteristic of yeast mitochondria. C: ^{35}S-labelled porin was presented to mitochondria isolated from the strains indicated. After incubation for 8 min at 25°C, mitochondria were extracted with alkali and collected after floatation through a sucrose density gradient. Porin inserted into the membrane was analysed by SDS–PAGE and quantitated by phosphorimage analysis. D: Mitochondria were isolated from cells expressing GFP–$_N$Tom7$_C$ and an aliquot representing 100 μg of mitochondria incubated in isotonic buffer (−), in isotonic buffer with 50 μg/ml trypsin (+), or in hypotonic buffer with 50 μg/ml trypsin (+M) for 15 min on ice. The samples were subsequently analysed by SDS–PAGE and immunoblotting with antibodies recognising the GFP epitope of the Tom7 fusion protein, the outer membrane protein Tom20 or the intermembrane space cytochrome b_2 (Cyb2). E: Mitochondria were isolated from cells expressing $_N$Tom7$_C$–GFP and aliquots representing 300 μg of mitochondria analysed as described above.

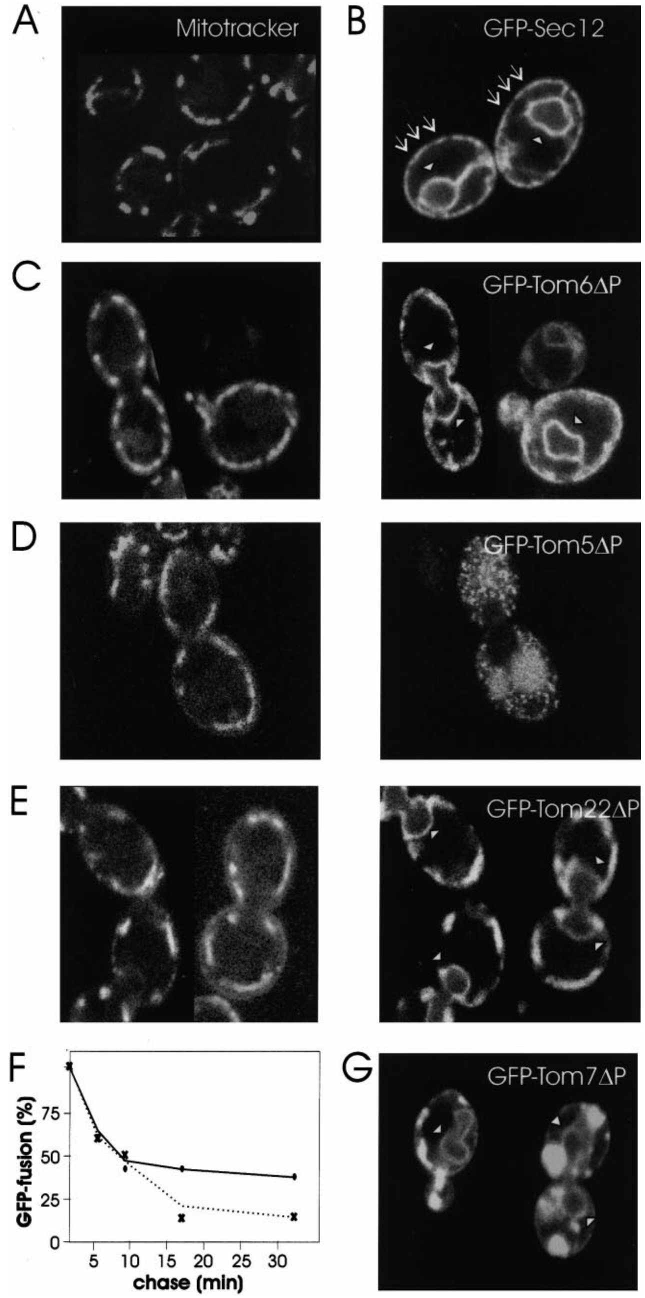

Fig. 3. A critical proline influences targeting of tail-anchored Tom proteins to mitochondria. Wild-type yeast cells were transformed to express the indicated GFP fusion proteins, co-stained with Mitotracker, analysed by fluorescence microscopy and a single optical section recorded. A: Wild-type cells stained with Mitotracker show the punctate cross-sectional profile of mitochondria. B: Cells expressing GFP–Sec12, a protein of the endoplasmic reticulum. Arrowheads indicate the perinuclear endoplasmic reticulum, arrows denote the peripheral endoplasmic reticulum. C: Cells expressing GFP–Tom6ΔP, co-stained with Mitotracker (left panel). D: Cells expressing GFP–Tom5ΔP, co-stained with Mitotracker (left panel). E: Cells expressing GFP–Tom22ΔP, co-stained with Mitotracker (left panel). F: Expression of GFP–Tom7 (straight line) or GFP–Tom7ΔP (dotted) in wild-type yeast cells. After pulse-labelling the cells with [^{35}S]methionine, samples were taken at the indicated times for immunoprecipitation with antisera recognising GFP and the immunoprecipitated GFP fusion protein analysed by SDS–PAGE and quantitated by phosphorimage analysis. G: Mutant *cim5-1* cells expressing GFP–Tom7ΔP.

In yeast, the information for targeting tail-anchored proteins to either the mitochondria or endoplasmic reticulum is found within a short segment of amino acids that includes the transmembrane segment [15]. This is also true for Tom7, since GFP–Δ6-28Tom7, consisting of only the last 33 amino acids of Tom7 (Fig. 2A), is still localised faithfully to mitochondria (data not shown).

3.3. A conserved proline residue is found in the transmembrane segments of the tail-anchored Tom proteins

A single proline residue is found in the transmembrane segment of each of the four tail-anchored Tom proteins: Tom5, Tom6, Tom22 and Tom7 (Fig. 3A). This residue is absolutely conserved across species in both Tom22 [16] and Tom7 (Fig. 1), the two proteins for which a large number of homologues have been identified during DNA sequencing projects. To address whether the proline residue is important for targeting these proteins to mitochondria, site-directed mutagenesis was used to replace the proline in each protein with a leucine residue.

In the case of Tom22(ΔP) and Tom6(ΔP), the replacement of the proline residue resulted in a proportion of the protein being targeted to the endoplasmic reticulum: the arrowheads in Fig. 3B,C,E,G indicate the perinuclear staining characteristic of the endoplasmic reticulum. In some cells, Tom5(ΔP) is further trafficked to the vacuole and associated vesicular structures (Fig. 3D). Co-staining of the cells with Mitotracker suggests a proportion of each protein is also associated with mitochondria (Fig. 3).

Fluorescence microscopy suggested Tom7(ΔP) was not stably expressed in wild-type cells, and pulse-chase analysis revealed the protein was turned over more rapidly than the wild-type protein (Fig. 3F). In *cim5-1* mutant cells, deficient in proteasome activity [17], Tom7 is localised exclusively to mitochondria (data not shown). Tom7(ΔP) was stably expressed in *cim5-1* cells at levels about 10-fold less than Tom7 (data not shown), but found targeted to both the endoplasmic reticulum and mitochondrial membrane (Fig. 3G).

4. Discussion

GFP–$_{N}$Tom7$_{C}$ is targeted to mitochondria and inserts in the outer membrane with tail-anchored topology [9]. The GFP fusion is functional, since it restores to *Δtom7* cells the ability to insert proteins in the outer membrane. $_{N}$Tom7$_{C}$–GFP is targeted to the mitochondria with the correct topology, but fluorescence in living cells is weak, suggesting incomplete folding of GFP. Work with GFP fusions to FtsZ and cytochrome *c* suggests GFP can fold in the intermembrane space of yeast mitochondria [18,19], but Tom7 is in direct contact with Tom40 [11], and both Tom40 and Tom22 have substantial domains exposed to the intermembrane space: in this crowded environment GFP might not fold effectively.

A previously overlooked feature of the tail-anchored proteins in the TOM complex is the presence of a single proline residue in the membrane-spanning domain. The residue is required for efficient targeting to mitochondria, since replacement with leucine residues renders each of the tail-anchored Tom proteins subject to insertion in the endoplasmic reticulum. However, a proline residue is not a critical determinant for targeting of other proteins to the mitochondrial outer membrane. Rather, the length and hydrophobic character of the transmembrane segment are critical [20], and the proline to leucine changes we made would alter the hydrophobic character of the targeting segment. In yeast, two of the other tail-anchored proteins are located in the mitochondrial outer membrane, Fis1 [21] and YFL046w (Beilharz and Lithgow, unpublished), yet neither has a proline residue in the predicted transmembrane segment. We suggest that while the hydrophilic nature of this proline residue is necessary for targeting, conserving a proline in this position may also have a rationale in terms of TOM translocase function.

Within a helical transmembrane segment the proline might produce a kink, as has been observed in the transmembrane segments of subunits in several ion channels and the *c* subunit of F_1F_0 ATP synthases [22]. In the case of the F_1F_0 ATPase, the *c* subunits thereby form a splayed barrel in the mitochondrial inner membrane [23]. Within the framework of the membrane-embedded TOM complex, a bent helix in one of the tail-anchored subunits might provide a key mechanical lever or ratchet for the translocase, or allow the subunits to be better positioned against the convex surface of the channel.

In the striking case of Tom22, while there is conservation of sequence within and between a variety of organisms previously analysed [16], the only truly invariant residue in the membrane-spanning segment is this unique proline, always found at position +15 with respect to the *cis* membrane surface (P^{112} in the *S. cerevisiae* sequence). Similarly in the case of each of the Tom7 homologues we found a proline residue (P^{43} in the *S. cerevisiae* sequence) is present at position +8 with respect to where the *cis* surface of the membrane is predicted to be. Whatever the precise function, conservation of this residue in each tail-anchored subunit in all plants, animals and fungi may reflect a critical feature of the TOM complex.

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