Identification of a third yeast mitochondrial Tom protein with tetratrico peptide repeats

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Abstract **The mitochondrial outer membrane contains a protein complex with at least eight subunits responsible for recognition and translocation of preproteins synthesized in the cytosol. Two subunits, the receptors Tom20 and Tom70, contain tetratrico peptide repeats that are thought to be involved in protein-protein interactions. We have identified *Saccharomyces cerevisiae* Tom72, a new Tom protein expressed at a low level. Tom72 is homologous to Tom70, including seven tetratrico peptide repeats. Tom72 is targeted to the mitochondrial outer membrane, forms a large domain exposed to the cytosol and loosely associates with the translocase complex of the outer membrane. These results suggest that Tom72 represents a ninth, weakly expressed component of the preprotein translocase of the mitochondrial outer membrane.**

Key words: Mitochondria; Translocase of outer mitochondrial membrane; Protein sorting; Import receptor

1. Introduction

Most mitochondrial proteins are synthesized as precursors on cytosolic polysomes and post-translationally imported into the organelle. The preproteins bind to receptors on the mitochondrial surface and are subsequently translocated across the mitochondrial membranes [1–4]. Four proteins of the outer membrane were found to be involved in recognition of preproteins and are assumed to form two receptor subcomplexes, Tom70-Tom37 and Tom20-Tom22. The receptors assemble together with several other outer membrane proteins, Tom40, Tom7, Tom6 and Tom5, to form a dynamic protein complex. Tom40 represents the main component of the general import pore. The small Tom proteins modulate the dynamics of the complex and may also be involved in formation of the import pore [5].

The molecular mechanisms of action of the Tom proteins are only partially understood. Tom22 contains a large number of negatively charged amino acid residues that are thought to interact with the positively charged mitochondrial targeting sequences by electrostatic interactions [6–8]. Tom20 and Tom70 contain tetratrico peptide repeats (TPR motifs), degenerate 34-residue sequences that are thought to participate in dynamic intramolecular and intermolecular protein-protein interactions [9,10]. The single TPR motif of Tom20 was shown to participitate in a loose interaction between Tom20 and Tom70 [11].

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Abbreviations: Tom, translocase of outer mitochondrial membrane (new uniform nomenclature [4]); Tom70 was formerly termed Mas70 or Mom72; Tom40 was termed Isp42 or Mom38; Tom20 was termed Mas20 or Mom19; TPR, tetratrico peptide repeat.

Since the Tom proteins are only loosely associated in the translocase complex [5] it cannot be excluded that further subunits of the complex exist. We report here that an open reading frame on *S. cerevisiae* chromosome VIII is expressed and encodes a protein with homology to Tom70. The protein Tom72 contains seven TPR motifs like Tom70 and is targeted to the mitochondrial outer membrane. Tom72 seems to represent a ninth subunit of the translocase of the outer mitochondrial membrane.

2. Materials and methods

2.1. Construction of plasmids and yeast mutants

For in vitro synthesis and expression in *E. coli* the coding region of the *TOM72* gene (open reading frame YHR117w of *S. cerevisiae* chromosome VIII [12]) was amplified by PCR using 1 μg of yeast genomic DNA. The amplification was performed with the sense primer 5'-GGATCCCATATGGCCGAAAACTCCCTCCTGA-3' with a *Bam*HI site and an *Nde*I site and the antisense primer 5'-CTGCAGCTCGAGCTAAAGCATGCCTTTAGCCCTATA-3' with a *Pst*I and an *Xho*I site. The amplified DNA fragment was ligated into the TA-cloning vector pCRII (Invitrogen) resulting in pCRII-*TOM72*. The accuracy of the amplified sequence was confirmed by double strand sequencing. The *Bam*HI-*Pst*I fragment of *TOM72* isolated from pCRII-*TOM72* was ligated in the *Bam*HI-*Pst*I linearized vector pGEM4Z (Promega), resulting in pGEM4Z-*TOM72*. To express *TOM72* in *E. coli* an *Nde*I-*Xho*I fragment of *TOM72* was isolated from pCRII-*TOM72* and ligated into the *Nde*I-*Xho*I linearized vector pET19b (Novagen), resulting in pET19b-*TOM72*. Tom72 with a $(His)_{10}$-tag at the N-terminus was purified by Ni-nitrilotriacetate affinity chromatography according to the manufacturer's instruction (Novagen) and used to generate antisera in rabbits.

To delete the coding region of the *TOM72* gene, a 600 bp fragment of the 5'-noncoding region was amplified using the sense primer 5'-ATAAGAATTCGGATACCACAAATACCAACAGTG-3' with an *Eco*RI site and the antisense primer 5'-ATAACTGCAGCCCGGGAAGAGCTTCTTATGTTCGGTATATA-3' with a *Pst*I site and an *Sma*I site. The amplified fragment was cleaved with *Eco*RI and *Sal*I and ligated into an *Eco*RI-*Sma*I linearized vector pGEM4Z, resulting in pGEM4Z-5'nonc. A 400 bp fragment of the 3'-noncoding region of *TOM72* was amplified using the sense primer 5'-ATAACTGCAGAAGATATGTCAGGTATTGGTCAAAT-3' containing a *Pst*I site and the antisense primer 5'-CCCGGGAAGCTTGTGGGTTAGTATTAAATTGTACATT-3' with a *Hin*dIII site. The amplified fragment was cleaved with *Pst*I and *Hin*dIII and subcloned into the *Sma*I-*Sal*I linearized plasmid pGEM4Z-5'nonc, resulting in pGEM4Z-5'+3'nonc. An *Sma*I-*Pst*I fragment encoding the *LEU2* gene was ligated into the *Sma*I-*Pst*I site of pGEM4Z-5' + 3'nonc, resulting in pGEM4Z-*tom72*Δ. The plasmid was linearized with *Hin*dIII and transformed into the diploid strain YPH501. Leucine-prototrophic cells were subjected to spore analysis. Deletion of the coding region of the *TOM72* gene was confirmed by PCR analysis. The *S. cerevisiae* strains used are listed in Table 1.

For generation of an *S. cerevisiae* strain deficient in *TOM70* and *TOM72*, heterozygous diploids were constructed by mating the strain MM208 (*tom70*Δ) [13] and KD30 (*tom72*Δ). After sporulation of the diploid cells, the genotypes of the spores were analyzed by growth on selective media. Standard procedures were used for manipulations of DNA and yeast strains [14,15].

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2.2. Mitochondria and protein import

Standard procedures (summarized in [16,17] and references therein) were used for growth of *S. cerevisiae*; isolation of mitochondria; treatment of mitochondria at alkaline pH (pH 11.5); trichloroacetic acid precipitation in presence of sodium deoxycholate; synthesis of preproteins in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and [^{35}S]cysteine; analysis by SDS-PAGE, Western blotting, scanning densitometry, and autoradiography using a storage phosphor imaging system (Molecular Dynamics). Total yeast cell extracts were prepared by heating in sample buffer according to Horvath and Riezman [18] except that the centrifugation of the extracts was omitted.

For pretreatment with protease, mitochondria (0.5 mg/ml) were incubated in SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2) with proteinase K (40 μg/ml) for 15 min at 0°C. Import reactions were performed at 25°C in import buffer (250 mM sucrose, 5 mM $MgCl_2$, 5 mM sodium malate, 2 mM ATP, 20 mM KP_i, 10 mM MOPS/KOH, pH 7.2). For co-immunoprecipitations import was performed in import buffer including 3% bovine serum albumin for 1 h. After a washing step with SEM, pelleted mitochondria were lysed in digitonin-containing buffer (0.5% digitonin, 250 mM sucrose, 10% glycerol, 3% BSA, 130 mM NaCl, 1 mM EDTA, 10 mM MOPS/KOH pH 7.2) and centrifuged for 15 min at 20000×g to remove unsolubilized material. Supernatants were subjected to co-immunoprecipitation with antibodies prebound to protein A-sepharose [17].

3. Results and discussion

The open reading frame YHR117w of *S. cerevisiae* chromosome VIII [12] encodes a putative protein of 639 amino acids (71.9 kDa) with similarity to Tom70. We show below that the open reading frame is expressed and that, according to the new uniform nomenclature for mitochondrial import components [4], the protein is named Tom72. It shows a similarity of 70% to *S. cerevisiae* Tom70, including 49% identical residues (Fig. 1A). The similarity to *N. crassa* Tom70 is 50%, including 28% identical residues. Like *S. cerevisiae* and *N. crassa* Tom70, Tom72 contains a hydrophobic segment at the N-terminus that is of sufficient length to function as membrane anchor sequence (residues 15–34; Fig. 1A).

A sequence analysis indicated that Tom72 contains TPR motifs (Fig. 1B). TPR motifs are degenerate 34-residue consensus sequences that are predicted to form two amphipathic α-helical subdomains. TPR motifs are thought to interact by a 'knob and hole'-like mechanism [9,10]. TPR motifs were previously found in various proteins involved in cell cycle control, transcription repression, stress response, neurogenesis, peroxisomal transport and in two Tom proteins, Tom70 and Tom20. Tom72 contains seven TPR motifs like Tom70 (Fig. 1B). One TPR motif of Tom72 (no. 6) and two TPR motifs of *N. crassa* Tom70 (nos. 3 and 6) are imperfect, i.e. deviate from the 34-residue length due to insertions as was previously observed for some other TPR-containing proteins [9]. A comparison of all TPR motifs of Tom72, *S. cerevisiae* Tom70 and *N. crassa* Tom70 (evolutionary distances) indicates a higher similarity between TPR motifs at the same position of the three proteins than between TPR motifs at different positions of the same protein. This suggests that the number and properties of the seven TPR motifs were determined in an ancestor of Tom70/Tom72 before the divergence of *N. crassa* and *S. cerevisiae* and before the gene duplication in *S. cerevisiae*.

The coding region of *TOM72* was cloned into the vector pET19b (Novagen) and expressed in *Escherichia coli* with a polyhistidine tag. After denaturation with 8 M urea, the expressed Tom72 was purified by immobilized metal affinity chromatography and injected into rabbits for generation of antibodies. Due to the similarity of Tom72 with Tom70, a cross-reaction of anti-Tom72 antibodies with Tom70 was expected. In a Western analysis of protein extracts from wild-type mitochondria, the anti-Tom72 antiserum indeed labeled two bands (Fig. 2A, lane 1). We thus tested the antiserum with *tom70Δ* mitochondria and found that only the faster migrating, weaker band was labeled (Fig. 2A, lane 2), indicating that the strong upper band represented Tom70 and the lower band represented Tom72. In agreement with this assignment, anti-Tom70 antibodies [19] strongly labeled the upper band (Tom70), yet only very weakly reacted with the Tom72 band. A treatment of mitochondria with protease degraded both Tom72 and Tom70 (Fig. 2A, lanes 5,6), indicating that both are exposed on the mitochondrial surface.

We constructed a yeast strain with a deletion of the *TOM72* gene. *tom72Δ* cells were viable and grew like wild-type cells with the exception of a slight reduction in growth rate on non-fermentable medium at higher temperature. By crossing with *tom70Δ* cells, we generated a double mutant *tom72Δ tom70Δ* that showed growth properties comparable to *tom70Δ* cells except of a slight enhancement of the growth reduction on non-fermentable medium at 37°C (*tom70Δ* cells show an about 2-fold reduction of the growth rate on non-fermentable medium at 37°C compared to wild-type cells). Thus, the *TOM72* gene is not essential for viability of *S. cerevisiae*. Its deletion leads to a slight growth defect on non-fermentable medium at higher temperature both in the presence and in the absence of *TOM70*.

The specificity of the antibody reaction was confirmed with mitochondria isolated from *tom72Δ* cells and *tom72Δ tom70Δ* cells. The lower band (Tom72) was lacking in *tom72Δ* mitochondria (Fig. 2A, lane 3) and both bands (Tom72 and Tom70) were lacking in mitochondria from the double mutant (Fig. 2A, lane 4). The recognition of Tom72 and Tom70 was tested with anti-Tom72 antibodies from various bleedings. In the assumption that anti-Tom72 antibodies from each bleeding recognize Tom72 at least as well as Tom70, the abundance

Table 1
S. cerevisiae strains used in this study

Strain	Genotype	Source
PK82	*MATα his4-173 lys2 ura3-52 Δtrp1 leu2-3,112*	[26]
YPH501	*MATa/α ade2-101/ade2-101 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 ura3-52/ura3-52 trp1-Δ63/trp1-Δ63 lys2-801/lys2-801*	[27]
YPH499	*MATa ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801*	[27]
MM208	*MATa ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom70::TRP1*	[13]
KD30	*MATα ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom72::LEU2*	this study
KD31	*MATα ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom70::TRP1 tom72::LEU2*	this study

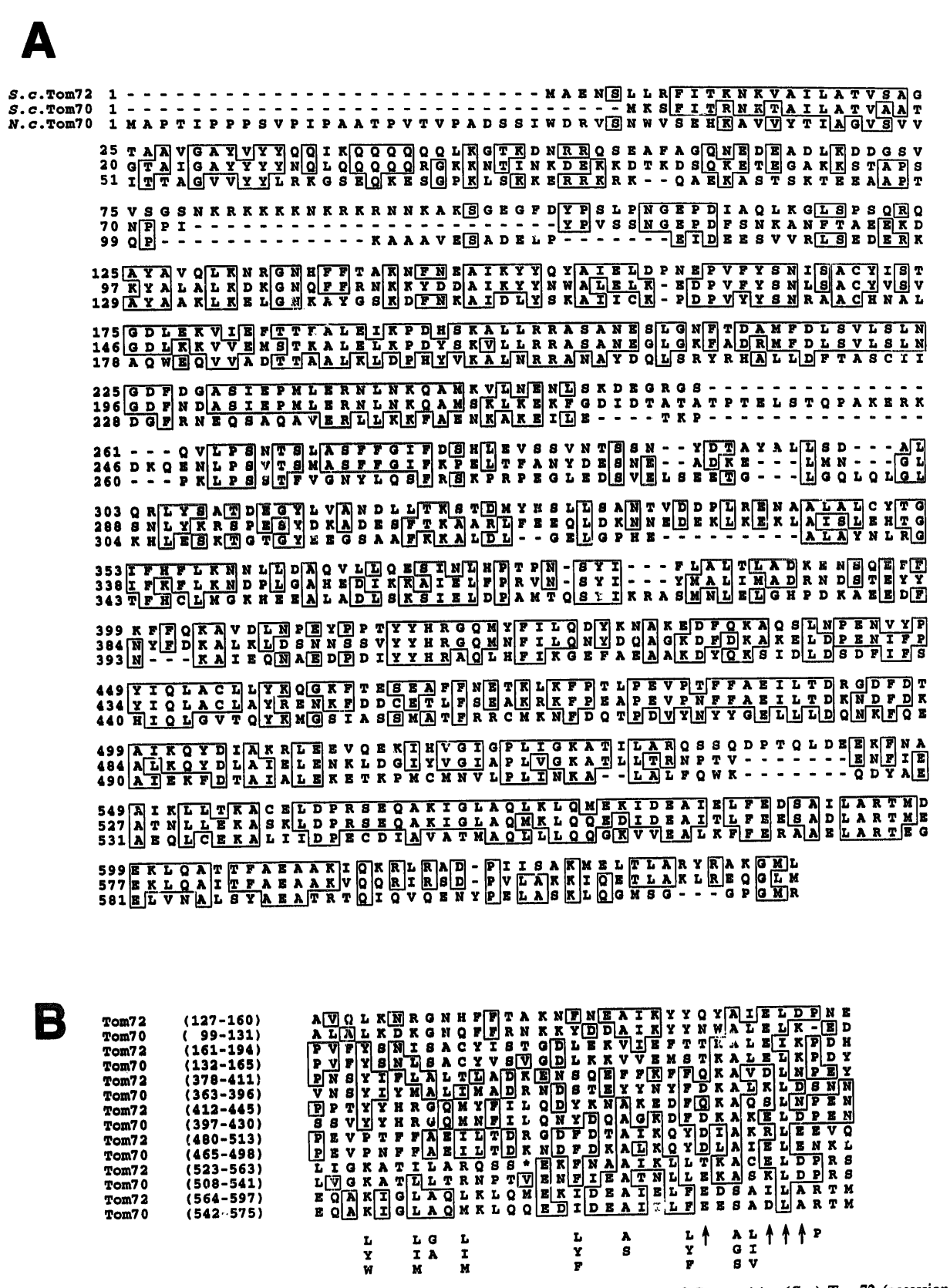

Fig 1. Tom72 contains seven TPR motifs. (A) Comparison of the predicted primary sequences of *S. cerevisiae* (*S.c.*) Tom72 (accession no. U00059), *S. cerevisiae* Tom70 (accession no. X05585) and *N. crassa* (*N.c.*) Tom70 (accession no. X53735) according to the algorithm of Hein [25]. Identical residues are boxed. (B) Comparison of the TPR motifs of Tom72 and Tom70 from *S. cerevisiae*. The asterisk indicates an insertion (amino acid sequence QDPTQLDE). General consensus residues of TPR motifs [9,10] are shown at the bottom; arrows indicate residues that are additionally conserved in the TPR motifs of *S. cerevisiae* Tom72 and Tom70.

of Tom72 in mitochondria is at least 8–10-fold lower than that of Tom70. A similar ratio was found when total cellular protein extracts were labeled with anti-Tom72 antibodies. A low level expression of *TOM72* was also suggested by Northern analysis of mRNA both in wild-type and *tom70Δ* cells (not shown). Consistently, the codon adaptation index [20] of *TOM72* is only 0.158, whereas the index of *TOM70* is 0.274.

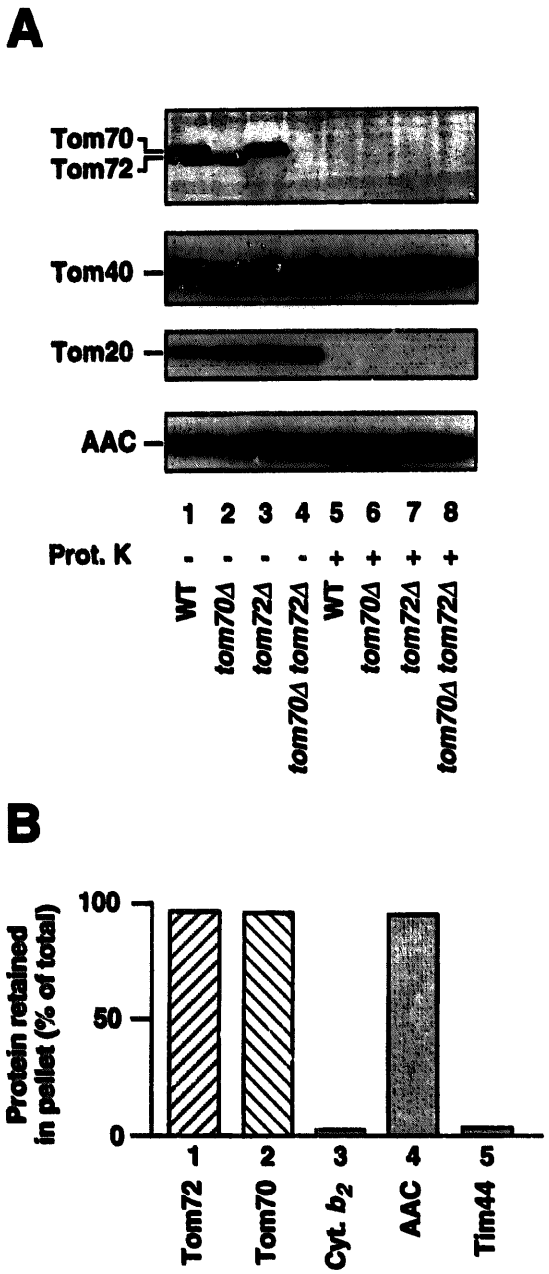

Fig. 2. Identification of Tom72 on purified mitochondria. (A) Tom72 is exposed on the mitochondrial surface. Mitochondria isolated from wild-type (lanes 1,5), *tom70Δ* (lanes 2,6), *tom72Δ* (lanes 3,7), or *tom70Δ tom72Δ* (lanes 4,8) *S. cerevisiae* cells were separated by SDS-PAGE and immunodecorated with antibodies directed against Tom72, Tom40, Tom20, or the ADP/ATP carrier (AAC). In samples 5–8, the mitochondria were treated with proteinase K. (B) Tom72 is not extracted at alkaline pH. Isolated mitochondria were treated with 100 mM Na_2CO_3 (pH 11.5). Pellets and supernatants were separated by centrifugation and analyzed by SDS-PAGE and immunodecoration with antibodies directed against Tom72, Tom70, cytochrome b_2 (Cyt. b_2), AAC, and Tim44.

A treatment of organelles with sodium carbonate at pH 11.5 can be used to distinguish between integral membrane proteins and soluble proteins/peripheral membrane proteins [21]. Soluble proteins, e.g. cytochrome b_2 of the mitochondrial intermembrane space (Fig. 2B, column 3), and peripheral membrane proteins, e.g. the mitochondrial inner membrane protein Tim44 (Fig. 2B, column 5), are extracted, whereas integral membrane proteins, e.g. outer membrane Tom70 and the ADP/ATP carrier of the inner membrane (Fig. 2B, columns 2 and 4) remain in the membrane sheets. Tom72 was not extracted at pH 11.5 (Fig. 2B, column 1), indicating that it was embedded in the lipid phase of the membrane.

The coding region of *TOM72* was cloned into the plasmid pGEM4Z. By in vitro transcription and translation in rabbit reticulocyte lysate, the precursor of Tom72 was synthesized in the presence of [^{35}S]methionine and [^{35}S]cysteine. The precursor was incubated with isolated mitochondria and associated with the mitochondria (Fig. 3A, upper panel, lanes 1,2). The efficiency of binding of the Tom72 precursor to mitochondria was higher than 90% of the amount of added precursor (Fig. 3, compare lanes 1 and 2 of the upper panel (mitochondria-associated Tom72) with that of the lower panel (Tom72 remaining in the reticulocyte lysate after reisolation of mitochondria)). A pretreatment of the mitochondria with proteinase K decreased the import of Tom72 by approx. 65% (Fig. 3, upper panel, lanes 3,4), indicating that a proteinase-sensitive component on the mitochondrial surface is required for its import, as was previously found for the precursor of yeast Tom70 [22]. Mitochondria with imported Tom72 were treated with trypsin after the import reaction, leading to a degradation of Tom72 (Fig. 3A, upper panel, lanes 5–8) and formation of a protease-resistant 60 kDa fragment that was released to the supernatant (Fig. 3A, lower panel, lanes 5–8). Thus, Tom72 forms a large protease-resistant cytosolic domain like Tom70 [23].

The precursors of Tom72 and Tom20 were synthesized and labeled in reticulocyte lysate and imported into isolated mitochondria. The mitochondria were then lysed in digitonin and subjected to co-immunoprecipitation with antibodies directed against Tom40, the central subunit of the outer membrane translocase complex, and, as control, with antibodies directed against porin, the most abundant outer membrane protein not involved in protein import. As expected, labeled Tom20 was found in association with Tom40, but not porin (Fig. 3B, lower panel, lanes 1,2) [24]. Labeled Tom72 was also found in association with Tom40 (Fig. 3B, upper panel, lanes 1,2), yet the yield of co-precipitation was only about 10% of that observed with Tom20. This agrees with observations made for Tom70; the majority of mitochondrial Tom70 is not stably associated with the translocase complex as the efficiency of co-precipitation of Tom70 with anti-Tom40 antibodies represents only 10–15% of that of Tom20 [5].

In conclusion, we have identified a new component of the translocase complex of the mitochondrial outer membrane of *S. cerevisiae*. Tom72 is homologous to Tom70, including an N-terminal hydrophobic sequence and a large cytosolic domain with seven TPR motifs. The position-specific conservation of the TPR motifs in Tom72, *S. cerevisiae* and *N. crassa* Tom70 indicates a functional or structural importance of the motifs. Tom72 is expressed in only low amounts (approx. 10-

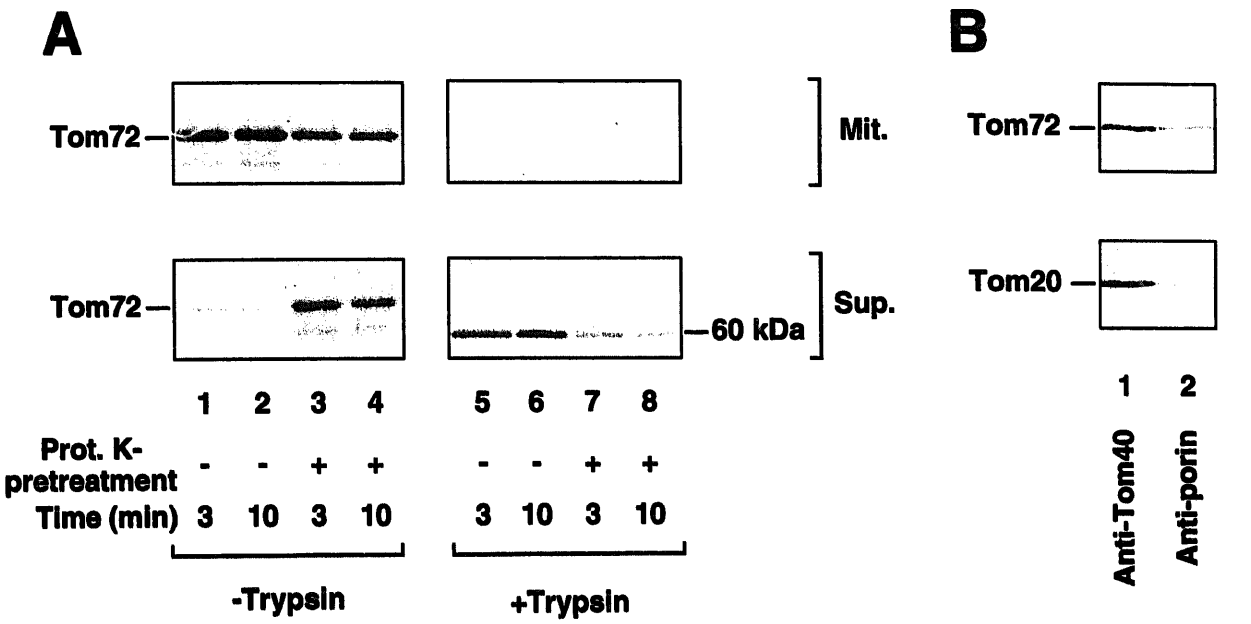

Fig. 3. Targeting of Tom72 to the yeast mitochondrial outer membrane. (A) Binding of Tom72 to isolated mitochondria and removal of a 60 kDa domain by trypsin. Rabbit reticulocyte lysate containing ^{35}S-labeled precursor of Tom72 was incubated with isolated yeast mitochondria at 25°C for the indicated times; the mitochondria of samples 3,4,7 and 8 had been pretreated with proteinase K to remove the surface receptors. The mitochondria were reisolated, and one half of the supernatant was precipitated by trichloroacetic acid. The mitochondria were washed and resuspended. One half of each sample was treated with trypsin (20 μg/ml), the other half was mock-treated. The mitochondria were reisolated, and pellets and supernatants (trichloroacetic acid-precipitated) were analyzed by SDS-PAGE and autoradiography. The upper panel shows the mitochondrial pellets (lanes 1–4, mock-treated; lanes 5–8, trypsin-treated). The supernatants are shown in the lower panel, that of the import reaction in lanes 1–4, that of the tryptic digest in lanes 5–8. In the mock-treatment, virtually no material was released to the supernatant (not shown). (B) Association of in vitro imported Tom72 and Tom20 with Tom40. Radiolabeled Tom72 and Tom20 were imported into isolated mitchondria. The mitochondria were washed, lysed by 0.5% digitonin, and the soluble material was subjected to co-immonupreciptation with antibodies directed against Tom40 (lane 1) or porin (lane 2). The amount of imported Tom72 subjected to co-immunoprecipitation was 5 times more than that of imported Tom20.

fold lower abundance than Tom70), it loosely associates with the outer membrane translocase in a manner reminiscent of Tom70. The molecular function of Tom72 is unknown. A deletion of *TOM70* does not lead to up-regulation of Tom72 and *vice versa*. Cells lacking the *TOM72* gene are viable both in the presence and in the absence of a functional *TOM70* gene; they show a slight impairment of growth only at higher temperature on non-fermentable medium, suggesting that Tom72 may be required for mitochondrial function under stress conditions.

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