

Role of Tom5 in Maintaining the Structural Stability of the TOM Complex of Mitochondria*

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Transport of nuclear encoded proteins into mitochondria is mediated by multisubunit translocation machineries in the outer and inner membranes of mitochondria. The TOM complex contains receptor and pore components that facilitate the recognition of preproteins and their transfer through the outer membrane. In addition, the complex contains a set of small proteins. Tom7 and Tom6 have been found in *Neurospora* and yeast, Tom5 has been found so far only in the latter organism. In the present study, we identified *Neurospora* Tom5 and analyzed its function in comparison to yeast Tom5, which has been proposed to play a role as a receptor-like component. *Neurospora* Tom5 crosses the outer membrane with its carboxyl terminus facing the intermembrane space like the other small Tom components. The temperature-sensitive growth phenotype of the yeast *TOM5* deletion was rescued by overexpression of *Neurospora* Tom5. On the other hand, *Neurospora* cells deficient in *tom5* did not exhibit any defect in growth. The structural stability of TOM complexes from cells devoid of Tom5 was significantly altered in yeast but not in *Neurospora*. The efficiency of protein import in *Neurospora* mitochondria was not affected by deletion of *tom5*, whereas in yeast it was reduced as compared with wild type. We conclude that the main role of Tom5, rather than being a receptor, is maintaining the structural integrity of the TOM complex.

Import of proteins from the cytosol into mitochondria is facilitated by several protein translocases, the TOM, TOB, and TIM complexes, located in the outer and inner mitochondrial membranes (1–6). In the outer membrane the TOM complex represents the main entry site for mitochondrial precursor proteins. It is a multimeric complex that mediates recognition of preproteins and their transfer across the outer membrane via a general transport pore.

Studies on the isolated TOM complex from *Neurospora crassa* and *Saccharomyces cerevisiae* have provided detailed

information about the structure and function of this translocase (7–12). The holocomplex consists of two receptor subunits, Tom70 and Tom20, and the proteins Tom40, Tom22, Tom7, and Tom6. The receptor subunits recognize different subsets of mitochondrial precursor proteins, whereas the other components form the protein-conducting channel complex (13–17). The TOM core complex consists of the subunits Tom40, Tom22, Tom7, and Tom6 (8).

An additional small subunit of the TOM complex, Tom5, was identified in *S. cerevisiae* (18). This protein is associated with the protein-conducting channel. Yeast mitochondria lacking Tom5 were reported to have severe defects in the import of proteins destined for the outer membrane (19), intermembrane space (20), inner membrane, and matrix compartment (18). This was taken to suggest that Tom5 is crucial for protein import to all mitochondrial subcompartments.

In yeast mitochondria, Tom5 is anchored in the outer membrane by a single transmembrane segment. Its primary structure revealed a cytosolic domain that carries a net negative charge. This feature led to the hypothesis that Tom5 guides positively charged mitochondrial targeting sequences from the surface receptors to the protein-conducting channel (18). A detailed structural analysis of the cytosolic domain of yeast Tom5 was performed using nuclear magnetic resonance spectroscopy. The cytosolic domain was found to exhibit an α -helical fold with alternating positive and negative charges (21). The charge topology did not present a pattern complementary to typical amino-terminal mitochondrial presequences. In addition, the structure did not provide a hydrophobic surface that could favorably interact with the hydrophobic face of amphiphilic mitochondrial presequences.

In view of the different concepts derived from functional and structural studies we decided to investigate the role of Tom5 in mitochondrial protein import in *Neurospora* and yeast. We cloned the *Neurospora* gene and tested whether Tom5 is interchangeable between *S. cerevisiae* and *N. crassa*. Although the amino acid sequence of *Neurospora* Tom5 shares only moderate sequence similarity with the yeast protein, it could substitute for its counterpart in yeast. In contrast to what was observed in yeast, *Neurospora* Tom5 deletion strains did not exhibit any growth defect. In line with this finding, mitochondria isolated from a *N. crassa* strain with an inactivated *tom5* gene were unimpaired in their capacity to import mitochondrial preproteins.

A role of Tom5 as a preprotein receptor was not observed in either organism. Rather our results point to a non-essential structural role of Tom5 in the TOM complex of *Neurospora*. In yeast, Tom5 appears to be required for maintaining the stability

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of the TOM complex at higher temperatures. This in turn is a prerequisite for functionality of preprotein import of the mitochondrial outer membrane protein translocation machinery.

MATERIALS AND METHODS

Strains and Growth Conditions—Growth and manipulation of *N. crassa* was performed as described previously (22). Standard procedures were used for the growth of *S. cerevisiae* cultures. The cells were grown at 30 °C and 37 °C on YP agar plates (1% (w/v) yeast extract and 2% (w/v) Bacto-peptone) containing either 2% (w/v) glucose (YPD) or 3% (v/v) glycerol (YPG).

Isolation of Mitochondria and Outer Membrane Vesicles—The preparations of mitochondria and mitochondrial outer membrane vesicles from *S. cerevisiae* and *N. crassa* were carried out as described previously (7, 23). Mitochondria used for protein import studies were isolated from the *N. crassa* wild-type strain 74A, the *N. crassa tom5^{RIP}* mutant strain, the *S. cerevisiae* wild-type strain YPH499, the *S. cerevisiae tom5Δ* mutant strain (a kind gift from Dr. N. Pfanner, University of Freiburg, Freiburg, Germany) and the *S. cerevisiae tom5Δ* strain rescued by a plasmid carrying the sequence information for a Fis1-Tom5C fusion protein (24).

Isolation of the TOM Core Complex—Large scale purification of TOM core complex was performed according to Ahting *et al.* (8) from *N. crassa* strain GR-107 that carries a hexahistidyl-tagged *tom22* gene instead of the wild-type copy (7). Mitochondrial membranes were solubilized for 30 min at 4 °C in 1% *n*-dodecyl β -maltoiside (Glycon Biochemicals), 50 mM potassium acetate, 10 mM MOPS, pH 7.2, 10% glycerol. After a clarifying by centrifugation the supernatant was passed through a Ni²⁺-nitrilotriacetic acid column (Qiagen), and the resin was washed with 40 mM imidazole. Specifically bound material was eluted with 300 mM imidazole, 50 mM potassium acetate, 10 mM MOPS, pH 7.2. For further purification, the TOM complex-containing fractions were pooled and transferred to a Resource Q anion exchange column (Amersham Biosciences) equilibrated with 50 mM potassium acetate, 10 mM MOPS, pH 7.2, 10% glycerol, 0.1% *n*-dodecyl β -maltoiside. TOM core complex was then eluted by a linear 0–500 mM KCl gradient. The protein solution was stored at 4 °C at a concentration between 2 and 5 mg/ml.

Mass Spectrometry—Individual subunits of isolated TOM core complex were separated by SDS-polyacrylamide gel electrophoresis. Tom5 was prepared for mass spectrometry by in-gel digestion according to procedures described previously (25–27). Peptides were dried and stored at –20 °C. Salts and detergents were removed by Zip-TipTM chromatography (C₁₈ reverse phase, Millipore). Wetting, equilibration of the column, peptide binding, washing, and elution of the peptides were performed as described in the Zip-TipTM user's manual.

All mass spectrometric analyses were carried out on a MicromassTM Q-TOF-1.5 hybrid mass spectrometer (Micromass, UK). The instrument was equipped with a z-electrospray ionization source and a quadrupole and a time-of-flight (TOF) mass analyzer as well as a hexapole collision cell. All spectra were acquired in electrospray positive ion mode. For sample introduction, a "medium" nanoelectrospray capillary was used (Micromass Q-TOF, Protana, Denmark). No electrospray ionization gas (nitrogen) or desolvation gas was applied to the capillary. The flow rate was regulated by alteration of the capillary voltage. The quadrupole was set in wide bandpass mode to 300–1500 *m/z*. Data were acquired in continuum mode. Duration of data collection was about 1 min. 25 scans were averaged at a rate of 2.0 s/scan. For electrospray MS/MS experiments, argon was used as collision gas, and the collision energy varied between 16 and 42 eV depending on the peptide analyzed. For representative spectra, data from 25–50 scans were summed. For optimized spectra the following conditions were applied: electrospray capillary voltage was set to 650–850 V; cone voltage was set to 45 V, and the microchannel plate was set to 2700 V when collecting MS data and to 2850 V for collecting MS/MS data. At first, the MS1 resolution was set to 10 to obtain optimal resolution. In later measurements the resolution was set to 15.

MS1 and MS2 were maintained as recommended in the user's guide. Acquired mass spectra were processed using software Mass-Lynx ver-

sions 3.4 and 3.5. For calibration, standard solutions of sodium iodide (2 μ g/ μ l) and rubidium iodide (0.05 μ g/ μ l) were dissolved in 50% propan-2-ol (Q-TOF user's guide). Calibration data were acquired over a mass range from 50 to 2000 *m/z*.

Cloning of *N. crassa tom5*—Based on parts of the Tom5 amino acid sequence, a 153-base genomic sequence was identified from the Whitehead Institute *Neurospora crassa* sequence data base (Cambridge, MA; *N. crassa* contig 3.132). A cDNA version of the *N. crassa tom5* gene was amplified from a λ Bluescript cDNA library by the PCR using T5_{up} (5'-CGCGGATCCCCGACTTCCATATCAAAATGTTCCG-3') and T5_{low} (5'-CCCAAGCTTCGAATATTTAGAGAACCTTGCTACGGC-3') as primers and cloned into pGEM-T (Promega). The PCR was performed using *Pfu* DNA polymerase (Promega) according to the manufacturer's instructions. DNA sequencing was performed using automated fluorescent sequencing technology (MediGenomix).

To improve the detection of [³⁵S]methionine-labeled Tom5 by autoradiography, a *tom5* gene encoding two additional methionine residues at the carboxyl terminus was cloned in pGEM-T with its expression under the control of the T7 promoter. The PCRs were carried out using T5_{up} and T5_{lowMM} (5'-CCCAAGCTTTTACATCATGAGAACCCTTGCTAACGGCGTCGATAACGAA-3') as primers. For expression of *N. crassa* Tom5 in *S. cerevisiae*, *N. crassa tom5* was cloned into the EcoRI/XhoI sites of pYX132 under the control of the constitutively active triose-phosphate isomerase promoter (Novagen). The construct and empty vector were transformed into yeast *tom5Δ* cells that were derived from the isogenic wild-type strain YPH-501 (18).

Antibodies—Antibodies against Tom5 were raised in rabbits using an oligopeptide corresponding to 20 amino-terminal amino acid residues of Tom5 as the antigen (NH₂-FQPPALSREELQAAEAEATF-COOH coupled to keyhole limpet hemocyanin; Pineda Antikörper Service, Berlin, Germany).

Construction of an *N. crassa* Strain Deficient in Tom5—The report of a severe phenotype in *tom5* mutants of *S. cerevisiae* (18) suggested that inactivation of *tom5* in *N. crassa* might also cause severe import and growth defects. For this reason, we chose to inactivate the gene using the procedure of "sheltered repeat-induced point mutation (RIP)." This procedure utilizes the natural phenomenon of RIP to inactivate genes in *N. crassa* (28) and also allows for the maintenance of nuclei containing non-functional alleles of an essential gene in a heterokaryon that also contains nuclei with a wild-type copy of the gene. The rationale and methodology of the procedure have been described previously (29). The *tom5* gene was found on chromosome I by examination of the sequence at the *N. crassa* genome sequencing website (www-genome.wi.mit.edu/annotation/fungi/neurospora). A duplication of *tom5* was created in the Host I strain by transformation with plasmid pTom5RIPhyg, which contains a 2.5-kb PCR product of the *tom5* region of the genome cloned into the hygromycin resistance vector pCSN44. The sheltered RIP cross-yielded several heterokaryotic strains. DNA from these strains was subjected to the PCR using primers specific for the *tom5* gene. Heterokaryon Tom5RIPed-35 was chosen as a strain that contained mutations characteristic of RIP in the *tom5* gene. Conidiospores from the heterokaryon were streaked onto medium containing all the requirements of the two nuclei that were components of the heterokaryon. Individual colonies were isolated and tested for nutritional requirements. Surprisingly many robust cultures were found to be nicotinamide-requiring, a trait that was diagnostic for the nucleus predicted to carry *tom5* genes that had been inactivated by RIP. Analysis of these cultures with antiserum raised against Tom5 revealed a total absence of the protein. Strain Tom5RIPed-35-5 (abbreviated *tom5^{RIP}*) was chosen for further work.

Cross-linking—For cross-linking experiments outer membrane vesicles (50 μ g) and purified TOM core complex (1.5 μ g) were suspended in 25 μ l of 50 mM HEPES, pH 7.2, and incubated with 0.1–0.4% (v/v) freshly prepared paraformaldehyde at 25 °C for 20–40 min. Aliquots were removed before and after the addition of the cross-linking reagent. Proteins were directly analyzed by SDS-PAGE and Western blotting.

Size Exclusion Chromatography—Outer membrane vesicles (100 μ g) isolated from wild-type and *tom5^{RIP}* mitochondria were solubilized in 0.2% *n*-dodecyl β -maltoiside with 50 mM potassium acetate, 10 mM MOPS (pH 7.2), 10% glycerol. After clarifying by centrifugation, detergent-solubilized proteins were loaded onto a Superose 6 PC 3.2/20 size exclusion column (Amersham Biosciences) equilibrated with the solubilization buffer using the Ettan-LC chromatography system (Amersham Biosciences). Protein was eluted at room temperature at a flow rate of 0.04 ml min^{–1}. Individual fractions were analyzed by SDS-PAGE and immunoblotting. The molecular masses of TOM complexes were calculated using UV spectra of chromatographs of thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (155 kDa), and

¹ The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; Q-TOF, quadrupole time-of-flight; MS, mass spectrometry; RIP, repeat-induced point mutation; BN, blue native; PVDF, polyvinylidene difluoride; bis-Tris, 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)propane-1,3-diol; Su9, subunit 9; DHFR, dihydrofolate reductase; TIM, translocase of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane; TOB, protein complex essential for the topogenesis of mitochondrial outer membrane β -barrel proteins.

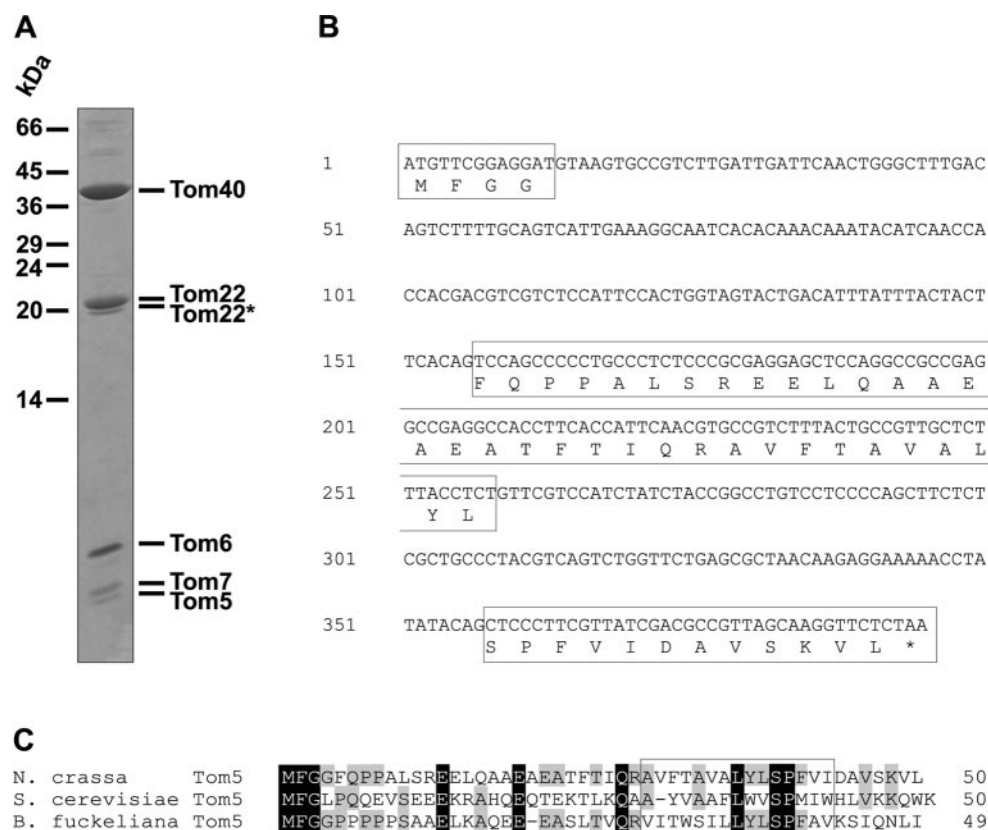


FIG. 1. Primary sequence of *N. crassa* Tom5. A, Coomassie-stained SDS-polyacrylamide gel of purified TOM core complex (50 µg) of *N. crassa*. The individual components of the multisubunit complex are indicated. Tom22* represents a degradation product of Tom22 lacking four amino-terminal amino acid residues. The position of molecular mass standards is indicated on the left. B, the amino acid coding sequence of Tom5 was deduced from fragments of isolated Tom5 by mass spectrometry and screening genomic and cDNA data bases of *N. crassa*. The coding region of the genomic DNA is interrupted by two introns. The protein coding regions are boxed. C, comparison of the amino acid sequences of *N. crassa* and *S. cerevisiae* Tom5 and an open reading frame of the fungus *B. fückeliana*. The proteins were aligned using the DNAMAN software (Lynnon BioSoft, Vaudreuil, Canada). Identical residues in all three proteins are shaded black; residues conserved in two of the proteins are in gray. The putative transmembrane segment is boxed.

carbonic anhydrase (29 kDa) as protein standards.

Stoichiometry—For the determination of the stoichiometry of Tom5, Tom6, and Tom7, the TOM core complex was isolated from strain GR-107 that had been grown in the presence of [³⁵S]sulfate as described previously (8). The purified TOM core complex was subjected to high Tris/urea SDS-polyacrylamide gel electrophoresis. Radiolabeled proteins were extracted from the gel by incubation of 1-mm gel slices in 10% SDS and quantified by scintillation counting (Tri-Carb 2100TR liquid scintillation analyzer, Canberra-Packard GmbH). The molar ratio between the individual subunits of the TOM complex was calculated based on their number of methionine and cysteine residues.

Import of Preproteins into Isolated Mitochondria—Preproteins were synthesized using the TnT® T7/SP6 coupled reticulocyte lysate transcription/translation system (Promega) in the presence of [³⁵S]methionine. *In vitro* transcription from pGEM4 vectors containing the gene of interest was performed with SP6 polymerase. Radiolabeled precursor proteins (2% reticulocyte lysate) were incubated at room temperature with 50 µg of *Neurospora* or *S. cerevisiae* mitochondria in 250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 13 mM ATP, 4.4 mM NADH, 3% (w/v) bovine serum albumin, 10 mM MOPS-KOH, pH 7.2, for 1–10 min. Import reactions were stopped by addition of ice-cold SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2). Mitochondria were reisolated by centrifugation for 10 min at 12,500 × g, resuspended in SEM buffer, and incubated with proteinase K for 15 min on ice followed by addition of 2 mM phenylmethylsulfonyl fluoride. Import was analyzed by SDS-PAGE and blotting to nitrocellulose membranes. Alternatively reisolated mitochondria were solubilized in 20 mM Tris, pH 7.2, 0.25% (w/v) *n*-dodecyl β-maltoside at 4 °C, subjected to blue native (BN)-PAGE and blotted to polyvinylidene difluoride (PVDF) membranes.

Gel Electrophoresis—BN-PAGE was carried out essentially as described previously (30). Mitochondria (50 µg) were solubilized in 100 mM bis-Tris, pH 7.2, 0.25% (w/v) *n*-dodecyl β-maltoside. After clarifying by centrifugation, the supernatants (40 µl) were supplemented with 5 µl of

sample buffer (5% (w/v) Coomassie Brilliant Blue G-250, 0.5 M ε-amino-*n*-caproic acid, 100 mM bis-Tris, pH 7.0) and electrophoresed through 6–13% polyacrylamide gradient gels. The cathode and anode buffers for performing the electrophoresis were as described previously (30).

SDS-polyacrylamide gel electrophoresis and blotting were performed according to standard procedures. To achieve a higher resolution of the smaller TOM components high Tris/urea gels were used (7). The chemiluminescence ECL detection system (Amersham Biosciences) and goat anti-rabbit antibodies conjugated to horseradish peroxidase were used for immunostaining. Radiolabeled precursor proteins were detected by autoradiography and quantified using the TINA software of the BAS 1500 phosphorimaging system (Fuji Medical Systems).

RESULTS

Identification of *Neurospora* Tom5—Analysis of purified TOM core complex of *Neurospora* by SDS-polyacrylamide gel electrophoresis revealed a previously unidentified subunit of the TOM complex of ~5-kDa molecular mass (Fig. 1A). To determine its amino acid sequence the protein was extracted from the gel and sequenced by mass spectrometry. Using parts of the amino acid sequence as queries, the genomic sequence encoding the protein was identified in the *N. crassa* nucleotide sequence data base (Fig. 1B). The *tom5* gene contains two introns and encodes a protein consisting of 50 amino acid residues. The Tom5 sequence can be viewed in GenBank™ under accession number CAE76411. The protein shares 34% sequence identity with Tom5 of *S. cerevisiae* and 44% sequence identity with an open reading frame of the fungus *Botryotinia fuckeliana*. The amino acid sequence of Tom5 exhibits a hydropathy profile with one predicted transmembrane segment near the carboxyl terminus, a feature conserved in the proteins

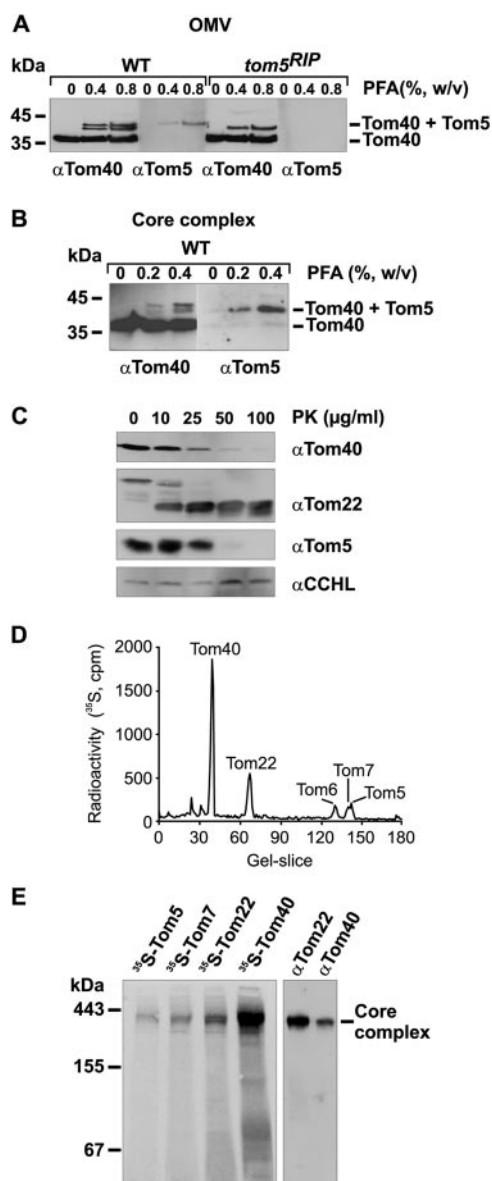


FIG. 2. Tom5 is a subunit of the *N. crassa* TOM complex and interacts with Tom40. Outer membrane vesicles (OMV) (A) and purified TOM core complex (B) isolated from wild-type (WT) and *tom5^{RIP}* mitochondria were incubated with 0–0.8% paraformaldehyde (PFA) in 25 μ l of reaction buffer (see “Materials and Methods”) for 20 min at 25 $^{\circ}$ C. The cross-link products were analyzed by SDS-PAGE and Western blotting using antibodies against Tom40 and Tom5. The second cross-link band below that of the Tom40 plus Tom5 product represents a cross-link between Tom40 and Tom6 (17). C, effect of proteinase K on TOM complex and components. Mitochondria (50 μ g) from wild-type and *tom5^{RIP}* cells were treated with 10–100 μ g/ml proteinase K (PK) or left untreated for 20 min on ice. Proteins were analyzed by SDS-PAGE, blotted to nitrocellulose, and decorated using antibodies against the amino-terminal domain of Tom5 (α Tom5), Tom40 (α Tom40), and Tom22 (α Tom22). A polyclonal antiserum against cytochrome *c* heme-lyase (α CCHL), a soluble intermembrane space protein (37), was used as a control for the integrity of the outer membrane. D, determination of the stoichiometry for Tom5 in isolated TOM complex. The TOM core complex was isolated from strain GR-107 that had been grown in the presence of [35 S] sulfate. The complex was subjected to high Tris/urea SDS-PAGE. Radiolabeled proteins were extracted from the gel by incubation of 1-mm gel slices in 10% SDS and quantified by scintillation counting. The activity of all gel slices is shown. E, 35 S-labeled precursor proteins of Tom5 were incubated for 20 min at 25 $^{\circ}$ C with 50 μ g of freshly isolated *N. crassa* mitochondria. As controls, mitochondria were incubated with the radiolabeled precursors of Tom7, Tom22, and Tom40. The mitochondria were isolated by centrifugation and solubilized in Tris buffer containing *n*-dodecyl β -maltoside. After clarifying by centrifugation, the supernatants of the samples were supplemented with sample buffer containing 5% Coomassie and 0.5 M ϵ -amino-

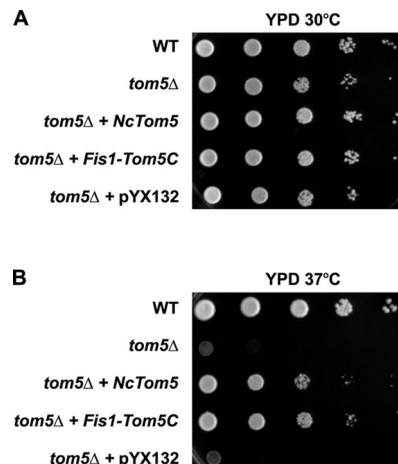


FIG. 3. Growth of yeast *tom5 Δ* cells transformed with *N. crassa* *tom5*. Dilution series of wild-type (WT, YPH499), *tom5 Δ* , and *tom5 Δ* yeast cells transformed with either *N. crassa* *tom5* (*tom5 Δ* + *NcTom5*) or a fusion of the cytosolic domain of Fis1 and the transmembrane segment of yeast Tom5 (*tom5 Δ* + *Fis1-Tom5C*) and cultivated on fermentable YPD medium at 30 $^{\circ}$ C (A) and 37 $^{\circ}$ C (B). As a control, *tom5 Δ* cells were transformed with the empty vector pYX132.

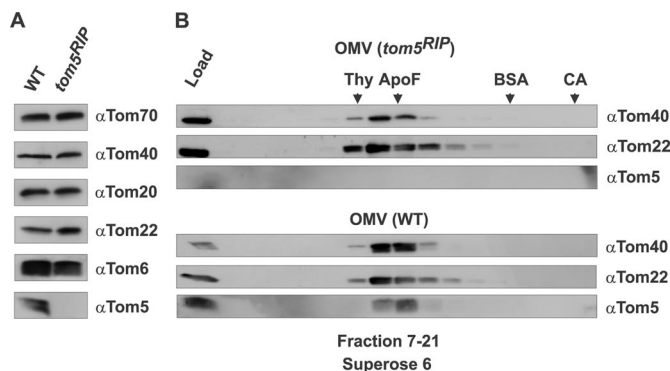


FIG. 4. Formation of the TOM core complex in the absence of Tom5. A, SDS-PAGE and immunoblot of mitochondria isolated from *N. crassa* wild-type and *tom5^{RIP}* cells. The blots were decorated with antibodies specific to the indicated components of the TOM complex. The expression level of *N. crassa* Tom7 could not be determined as no suitable antiserum was available. B, size exclusion chromatography of the TOM complex lacking Tom5. Outer membrane vesicles (OMV, 100 μ g) isolated from *N. crassa* cells deficient in Tom5 were lysed in solubilization buffer containing 0.5% *n*-dodecyl β -maltoside and subjected to gel filtration on a Superose 6 column. Fractions were analyzed for the presence of the TOM complex by SDS-PAGE and immunoblotting with antibodies recognizing Tom40 (α Tom40), Tom22 (α Tom22), and Tom5 (α Tom5). As a control, the same protocol was performed with outer membrane vesicles isolated from wild-type (WT) cells. The molecular mass markers were thyroglobulin (Thy, 669 kDa), apoferritin (ApoF, 443 kDa), bovine serum albumin (BSA, 66 kDa) and carbonic anhydrase (CA, 29 kDa).

of *B. fockeliana* and *S. cerevisiae* (Fig. 1C). The amino-terminal domain of *Neurospora* Tom5 contains four negatively charged amino acid residues. However, the positions of only two of them are conserved among the three organisms mentioned above.

N. crassa Tom5 Is Located Close to the Main Subunit of the Protein-conducting Channel Tom40—Treatment of outer membrane vesicles and of purified TOM core complex with the short

n-caproic acid, analyzed by BN-PAGE, and blotted to PVDF membranes. Radiolabeled precursors were detected by autoradiography. Individual subunits of the TOM complex were detected using antisera against *N. crassa* Tom22 (α Tom22) and Tom40 (α Tom40) and demonstrate that the complex comigrates with the incorporated radiolabeled precursors. The positions of molecular mass markers apoferritin (443 kDa), alcohol dehydrogenase (155 kDa), and bovine serum albumin (66 kDa) are shown.

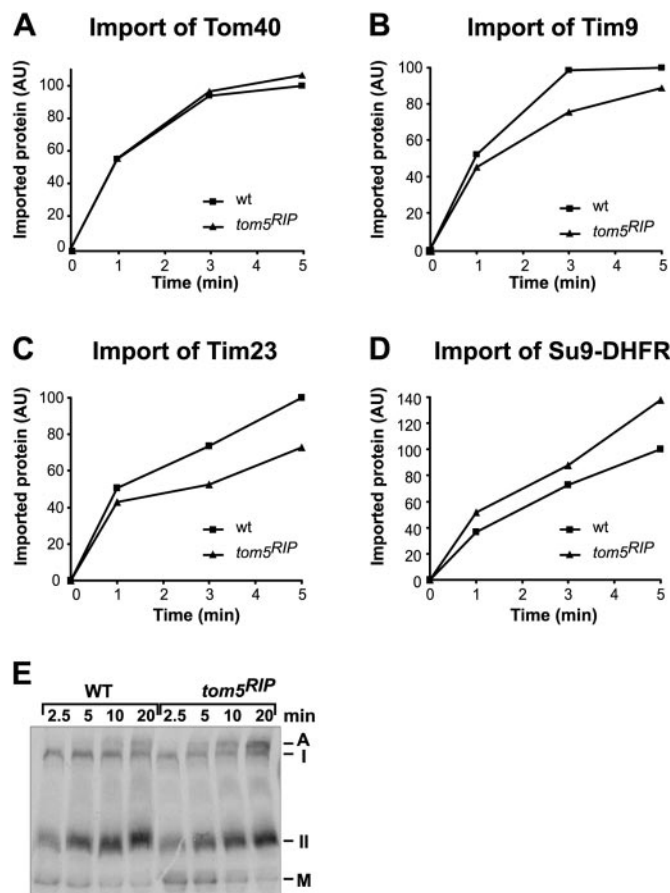


FIG. 5. Protein import into *N. crassa* mitochondria deficient in Tom5. Mitochondria were freshly isolated from the *N. crassa* *tom5*^{RIP} strain and a wild-type control strain. For protein import, [³⁵S]-labeled preproteins were incubated with isolated mitochondria (50 μ g) at 25 °C for the indicated times. After stopping the import reaction with ice-cold SEM buffer, the mitochondria were sedimented by centrifugation, resuspended in 100 μ l of SEM buffer, and treated with 25 μ g/ μ l proteinase K for 15 min on ice. The samples were analyzed by SDS-PAGE and blotted to nitrocellulose membranes. Protease-resistant protein was quantified by autoradiography. The following preproteins were used: Tom40 (A), Tim9 (B), Tim23 (C), and pSu9-DHFR (D). The import signal of wild-type mitochondria after 5 min was set to 100% import (arbitrary units (AU)). E, influence of the *tom5* deletion on the insertion pathway of Tom40. [³⁵S]Tom40 precursors were incubated at 25 °C with mitochondria (50 μ g) isolated from wild-type (WT) and *N. crassa* *tom5*^{RIP} strains for the indicated periods of time. Mitochondria were reisolated by centrifugation and lysed in a buffer containing 1% digitonin. After clarifying by centrifugation, the samples were subjected to BN-PAGE and blotted to PVDF membranes as described in Fig. 2. Radiolabeled precursors were detected by autoradiography. M, I, II, and A mark the monomeric form of Tom40, two Tom40 assembly intermediates, and the fully assembled TOM complex, respectively (6, 38).

length cross-linker formaldehyde yielded adducts between Tom5 and Tom40 (Fig. 2, A and B). This points to a direct contact between Tom5 and the main component of the protein-conducting channel, Tom40. Proteinase K efficiently degraded the large hydrophilic domain of Tom22 exposed to the cytosol, but Tom5 became accessible to degradation only at proteinase K concentrations higher than those required for degradation of Tom40 and Tom22 (Fig. 2C). Thus, Tom5 appears to be shielded by other Tom components. Furthermore we could not detect any fragments of Tom5. As the antibody recognizes an epitope within the amino-terminal part of the protein we conclude that the amino terminus is facing the cytosol.

To estimate the molar relationship of Tom5 to the other Tom components we purified TOM core complex from *N. crassa* cells that were grown in the presence of [³⁵S]sulfate. Analysis of isolated core complex by SDS-PAGE and scintillation counting

of extracted protein (Fig. 2D) yielded a molar ratio between Tom5 and the other Tom components Tom40, Tom22, Tom7, and Tom6 of roughly 1:1:1:1.

To confirm that the newly identified protein is a real subunit of the TOM complex radiolabeled Tom5 was imported into mitochondria isolated from *Neurospora*. As controls, we studied the integration of the radiolabeled precursors of Tom7, Tom22, and Tom40 into the TOM complex. Imported Tom5 was shown to be present in the mature 400-kDa TOM core complex (Fig. 2E). In contrast to imported Tom40 no low molecular mass import intermediate was observed indicating that Tom5 inserted into a largely assembled TOM complex.

Neurospora Tom5 Is a Functional Homolog of Yeast Tom5—To test whether *Neurospora* Tom5 can substitute for yeast Tom5 we transformed the *Neurospora tom5* cDNA into a yeast *TOM5* deletion mutant that was described to exhibit a characteristic temperature-sensitive growth defect (18). Yeast cells lacking *tom5* stopped growing when the temperature was raised from 30 to 37 °C (Fig. 3). Expression of *Neurospora* Tom5 in these cells restored growth. This indicated that the Tom5 proteins in the two fungi are functional equivalents.

Depletion of Tom5 in Neurospora Does Not Affect Growth—We used the procedure of sheltered RIP to inactivate the *tom5* gene (*tom5*^{RIP} strain) and found that homokaryotic strains lacking Tom5 could be derived from the sheltered heterokaryons. Mitochondria isolated from the homokaryon were analyzed by SDS-PAGE and immunoblotting. The expression levels of the other Tom components were similar in mutant and wild-type cells (Fig. 4A). The apparent molecular mass of the complex lacking Tom5 did not differ from that of the wild-type complex (Fig. 4B).

In contrast to what was reported for yeast, *Neurospora* cells deficient in Tom5 did not exhibit a growth defect at either 25 or 42 °C (data not shown). To determine whether a possible suppressor of the inactivated *tom5* gene was present in the *tom5*^{RIP} strain, we crossed it with the standard wild-type strain 74A. Of 36 random progeny examined, 19 were devoid of Tom5, and 17 had normal levels of the protein. No differences in growth rate between the Tom5-deficient strains and the normal strains were observed either from ascospores or during vegetative propagation (not shown). Thus, the possibility of a mutation in another gene suppressing the phenotypic consequences of the Tom5 deficiency was unlikely unless the suppressing gene was very closely linked to the *tom5* gene. The most probable suppressors of a Tom5 deficiency might be expected to be other components of the TOM complex that had been modified to compensate for the lack of Tom5 function. However, searches of the *N. crassa* genomic data base revealed that Tom5 is the only TOM complex protein encoded on chromosome I so that no other gene encoding a component of the complex is closely linked to the *tom5* gene. We also produced Tom5-deficient strains using unsheltered RIP by directly crossing the *tom5* duplication strain (*tom5*^{RIP}*hyg*) with the wild-type strain 74A. Two strains lacking Tom5 that were examined from this cross also showed no abnormalities of growth rate (data not shown). We conclude that the absence of Tom5 has no obvious consequences on the growth characteristics of the organism.

Depletion of Tom5 in Neurospora Has No Effect on Import of Mitochondrial Preproteins—To assess whether Tom5 functions as a receptor for mitochondrial preproteins, *in vitro* synthesized mitochondrial preproteins labeled with [³⁵S]methionine were imported into mitochondria isolated from *tom5*^{RIP} cells. Fig. 5 (A–D) shows the import kinetics of the mitochondrial outer membrane protein Tom40, the intermembrane space protein Tim9, the inner membrane protein Tim23, and the matrix-targeted protein pSu9-DHFR, consisting of the presequence of

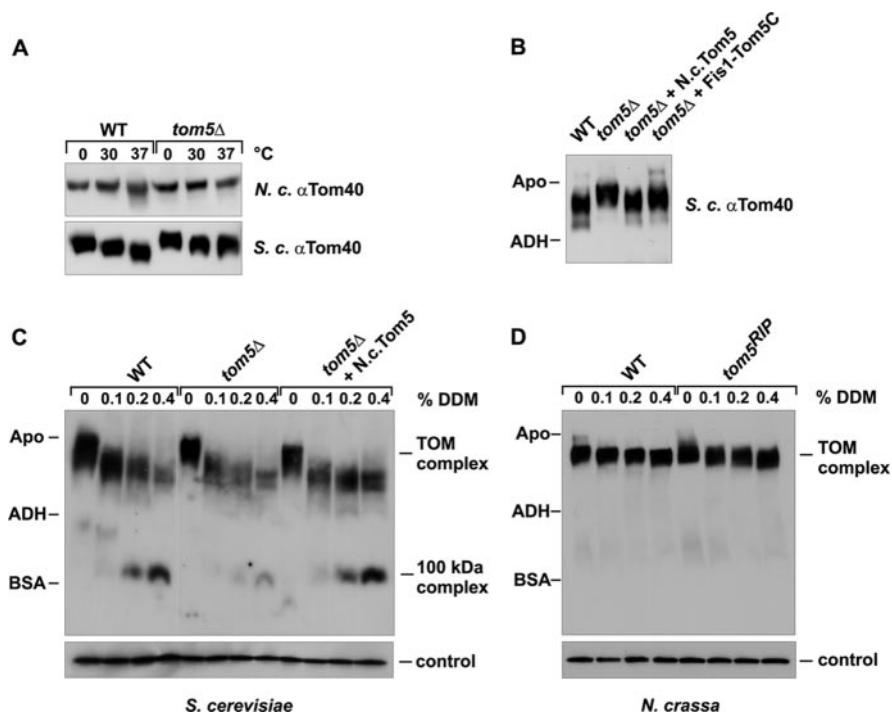


FIG. 6. **Stability of TOM complex in *N. crassa* and *S. cerevisiae*.** A, mitochondria of *N. crassa* wild-type and *tom5^{RIP}* cells and of yeast wild-type and *tom5Δ* cells were solubilized in digitonin at 0, 30, and 37 °C. The samples were analyzed by BN-PAGE and immunoblotting using antibodies against Tom40. B, comparison of the size of TOM complexes of different *S. cerevisiae* (S.c.) strains: wild-type (WT), *tom5Δ*, *tom5Δ* complemented by the *N. crassa* (N.c.) *tom5*, and *tom5Δ* complemented by a fusion protein existing of the cytosolic domain of Fis1 and the transmembrane region of Tom5 at the carboxyl terminus (24). Isolated mitochondria were solubilized in digitonin at 37 °C, subjected to BN-PAGE, blotted to PVDF membrane, and immunodecorated with an antibody against Tom40. Mitochondria isolated from wild-type, *tom5Δ*, and *tom5Δ* complemented by the *N. crassa tom5* yeast cells (C) and from *N. crassa* wild-type and *tom5^{RIP}* cells (D) were solubilized at 37 °C in a digitonin-containing buffer supplemented with increasing amounts of *n*-dodecyl β -maltoside as indicated. Samples were taken from the solubilized material and subjected to SDS-PAGE for a load control. After a centrifugation step equal amounts of the solubilized mitochondria were subjected to BN-PAGE. All gels were blotted to PVDF membranes and analyzed by immunodecoration against Tom40. BSA, bovine serum albumin; ADH, alcohol dehydrogenase, Apo, apoferritin.

subunit 9 (Su9) of the F_0 -ATPase and dihydrofolate reductase (DHFR). No significant differences were observed in the import kinetics of these precursors between mitochondria isolated from wild-type cells and cells lacking Tom5. The assembly of Tom40 into the TOM complex was not affected in Tom5-deficient mitochondria (Fig. 5E). We conclude that Tom5 has no direct role in the import of mitochondrial preproteins or in the biogenesis of the TOM complex in *N. crassa*.

Tom5 Is Required for the Thermal Stability of the TOM Complex of Yeast but Not of *Neurospora*—We compared the thermal stability of the TOM complex of *Neurospora* and yeast. Mitochondria were incubated at 0, 30, and 37 °C; solubilized with mild detergent; and subjected to BN-PAGE. The electrophoretic mobility of the *Neurospora* TOM complex, both wild type and Tom5-deficient, was not affected by this treatment (Fig. 6A, upper panel). In contrast, the mobility of the wild-type yeast TOM complex was strongly temperature-dependent. The effect increased with rising temperature indicating a structural reorganization. The absence of Tom5 affected the altered mobility of the yeast complex in the BN-PAGE analysis since the temperature shift from 30 to 37 °C did not cause a significant change of the electrophoretic mobility as it did for the wild-type complex (Fig. 6A, lower panel).

To confirm that structural alterations in the yeast complex were due to Tom5, we studied the electrophoretic mobility of TOM complexes of a mutant in which the protein was replaced by its *Neurospora* counterpart. In addition, we tested a complex in which only the carboxyl-terminal segment of yeast Tom5 (*i.e.* the transmembrane domain and the last four amino acids facing the intermembrane space) fused to the cytosolic domain of the model protein Fis1 (24) was present (Fis1-Tom5C). In

both cases the electrophoretic mobility of the TOM complex was similar to that of the wild-type yeast complex (Fig. 6B).

To compare the stability of yeast and *Neurospora* TOM complexes mitochondria from both organisms were solubilized in the presence of increasing concentrations of the non-ionic detergent *n*-dodecyl β -maltoside (Fig. 6, C and D). The TOM complex of *Neurospora* was stable up to 0.4% (w/v) *n*-dodecyl β -maltoside irrespective of the presence or absence of Tom5 (Fig. 6D). The TOM complex of *S. cerevisiae*, however, was found to dissociate into a subcomplex with a molecular mass of ~100 kDa (Fig. 6C). In the absence of yeast Tom5, the TOM complex was completely unstable. Most of its components aggregated even at higher detergent concentrations.

There was no difference between the apparent molecular mass of the TOM complex lacking Tom5 compared with the wild-type complex in *Neurospora* (see Figs. 4B and 6A, upper panel). In addition, the stability of the Tom5-deficient complex when exposed to increasing concentrations of *n*-dodecyl β -maltoside did not differ from that of wild-type complex (Fig. 6D). In electrophysiological analysis and electron microscopic studies of the isolated Tom5-deficient complex no differences were found in comparison with the wild-type complex (data not shown). In conclusion, in the absence of Tom5 the overall architecture of the *Neurospora* TOM complex remains unaltered.

The Carboxyl-terminal Segment of Tom5 Rescues the Protein Import in *S. cerevisiae tom5Δ* Cells—Finally we asked whether the transmembrane segment of yeast Tom5 can complement the temperature-sensitive growth defect of yeast cells lacking Tom5 and restore the ability of *tom5Δ* mitochondria to import proteins. As a model protein we used the chimeric protein Fis1-Tom5C (24). Protein import studies were performed using

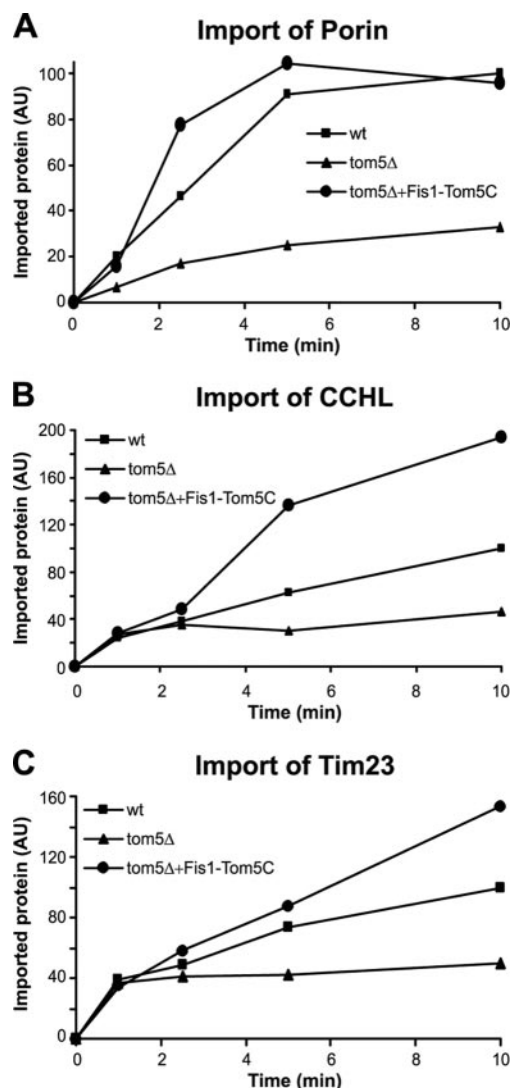


FIG. 7. **Protein import into *S. cerevisiae* mitochondria deficient in Tom5 rescued by Fis1-Tom5C.** Mitochondria were isolated from *S. cerevisiae* *tom5Δ* cells, *tom5Δ* cells expressing a fusion protein consisting of the amino-terminal domain of Fis1 and the transmembrane region of Tom5 (24), and wild-type (*wt*) cells. Protein import reactions were performed as described in Fig. 5 using radiolabeled preproteins. The samples were analyzed by SDS-PAGE and blotted to nitrocellulose membranes. Protease-resistant protein was quantified by autoradiography. The following preproteins were used: mitochondrial porin (voltage-dependent anion channel) (A), cytochrome *c* heme-lyase (CCHL) (B), and Tim23 (C). AU, arbitrary units.

in vitro synthesized mitochondrial preproteins labeled with [³⁵S]methionine and mitochondria isolated from wild-type, *tom5Δ*, and *tom5Δ* cells expressing Fis1-Tom5C.

Fig. 7 (A–C) shows the import kinetics of the outer membrane protein mitochondrial porin (voltage-dependent anion channel), the intermembrane space protein cytochrome *c* heme-lyase, and the inner membrane protein Tim23. Expression of Fis1-Tom5C largely restored protein import into mitochondria. According to this the temperature-sensitive phenotype of *tom5Δ* cells was efficiently complemented by expression of Fis1-Tom5C (Fig. 3).

DISCUSSION

Several studies on TOM complex-mediated protein translocation in *Neurospora* and yeast have indicated striking functional and structural similarities between the two evolutionary distant organisms (7–12). With the identification of Tom5 in *Neurospora* we provide further evidence that the

TOM complexes of *Neurospora* and yeast contain an identical set of subunits. Tom5 is part of the TOM core structure and tightly associated with the protein-conducting channel protein Tom40. The precursor of *Neurospora* Tom5 was imported into mitochondria and assembled into pre-existing TOM complexes in the same manner as its yeast counterpart (16). This supports the view that the TOM complex forms a dynamic structure in the mitochondrial outer membrane that can partially disassemble to allow the exchange of pre-existing subunits with newly imported precursor proteins (16, 31, 32). Yeast cells deleted in Tom5 do not grow at 37 °C either on fermentable or on non-fermentable carbon sources (18, 33). Expression of *Neurospora* Tom5 in the mutant yeast cells fully rescued the growth phenotype. This suggests that the Tom5 protein fulfils an equivalent function in the TOM complex of the two organisms. Data base searches so far did not reveal any mammalian Tom5 homologs. Yet a small molecular mass protein of ~5 kDa was co-immunoprecipitated with antibodies against Tom40 of rat (34). Therefore, a homolog to yeast and *Neurospora* Tom5 might be present in mammalian TOM complexes. Several proteins of low molecular mass associated with the TOM complex were detected in plants, but none has yet been identified as a Tom5 homolog (1, 35).

For yeast Tom5 a role as a general import receptor in protein import has been proposed (18). However, unlike in yeast, all tested precursor proteins of the various mitochondrial subcompartments were imported into *Neurospora* mitochondria deficient in Tom5 as effectively as into wild-type mitochondria. A role of yeast Tom5 in assembly of Tom40 into the TOM complex *in vitro* has also been reported (16). In the absence of Tom5 a radiolabeled Tom40 precursor accumulated in a 250-kDa assembly intermediate containing Tob55, and the maturation into the 400-kDa TOM complex was impaired (6, 16). In contrast, in a *tom5^{RIP}* strain of *Neurospora* we did not detect any defects in the assembly pathway of Tom40. Furthermore TOM5 is not an essential gene in yeast, and endogenous protein levels of Tom40 were not altered either in a yeast (18) or in a *Neurospora* Tom5 deletion strain. A receptor-like function of Tom5 would be expected to reside in its cytosolic domain. However, a fusion protein consisting of the transmembrane region of yeast Tom5 with the unrelated protein Fis1 replacing the cytosolic domain is sufficient to rescue the growth phenotype and the mitochondrial protein import defect of the yeast Tom5 deletion mutant (24, 33). This makes a receptor-like function of Tom5 unlikely.

As our findings did not agree with a receptor-like function of Tom5 we asked whether Tom5 rather has a structural role. The yeast TOM complex lacking Tom5 exhibited a decreased stability as compared with the complex from wild-type cells. Exposure of the yeast TOM complex to mild detergent caused it to disassemble to a 100-kDa species. In the Tom5 deletion mutant this species was absent, and most of the protein seemed to be aggregated. Integration of *Neurospora* Tom5 into the Tom5-deficient yeast TOM complex restored its stability. A structural role of Tom5 was also suggested by an altered electrophoretic behavior of the TOM complex from Tom5-lacking cells. Again this could be cured by integration of *Neurospora* Tom5 or likewise the expression of a fusion protein consisting of the cytosolic domain of Fis1 and the transmembrane segment of yeast Tom5. In *Neurospora* we did not observe differences in the electrophoretic mobility after heat treatment or upon exposure to detergent. We conclude that *Neurospora* TOM complex is more stable than its yeast counterpart and does not depend on the function of Tom5 under the conditions analyzed.

We suggest that the membrane-spanning segment of Tom5 is responsible for its structural function. This is supported by the

finding that the transmembrane region of Tom5 is sufficient to rescue the growth phenotype of the yeast Tom5 deletion mutant (24, 33). A proline residue within the transmembrane segment that was shown to be part of a targeting signal of Tom5 is conserved among the Tom5 proteins so far identified (33). In addition, a proline residue can be found in the transmembrane region of the Tom6, Tom7, and Tom22 proteins. This led to the hypothesis that it is essential for the interaction of these tail-anchored components of the TOM complex with the pore-forming protein Tom40 (24, 36). In conclusion Tom5 plays a role in maintaining the structural integrity of the TOM complex but has no apparent role as a presequence-recognizing receptor component.

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