Tom71, a Novel Homologue of the Mitochondrial Preprotein Receptor Tom70*

(Received for publication, December 29, 1995, and in revised form, March 28, 1996)

Jens Schlossmann, Roland Lill‡, Walter Neupert, and Deborah A. Court

From the Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Godthstrasse 33, 80336 München, Germany

The protein Tom71 is encoded by the open reading frame YHR117w (yeast chromosome VIII) and shares 53% amino acid sequence identity with Tom70, a protein import receptor of the mitochondrial outer membrane. We investigated the cellular function of Tom71 and addressed the question of whether Tom71 and Tom70 fulfill similar functions. Like Tom70, Tom71 is associated with the mitochondrial outer membrane via its N terminus, thereby exposing a large C-terminal domain to the cytosol. Tom71 is associated with the protein import complex of this membrane and can be cross-linked to a protein with a molecular mass of 30–35 kDa. Disruption of the TOM71 gene does not reduce cell growth, except on nonfermentable carbon sources at elevated temperatures. Deletion of both the TOM71 and TOM70 genes does not acerbate this growth defect. In vitro import studies demonstrated no functional requirement for Tom71 in the import of several preproteins destined for each of the mitochondrial subcompartments. In particular, the import of Tom70-dependent preproteins is minimally affected by the deletion of Tom71, irrespective of the presence or absence of the Tom70 receptor. Thus, despite their strikingly similar biochemical properties, Tom71 and Tom70 do not perform identical functions.

Most mitochondrial proteins are synthesized as precursors (preproteins) on cytosolic ribosomes and are then imported into the organelles (1–3). Recognition and translocation of these preproteins are facilitated by multi-subunit complexes in both the outer (TOM complex; Ref. 4) and inner membranes (TIM complex; Refs. 5 and 6). The TOM complex of both Neurospora crassa and Saccharomyces cerevisiae is composed of at least seven proteins (7–9). The membrane-embedded components are thought to comprise the general insertion pore through which preproteins traverse the outer membrane (10). These components include Tom40 (formerly termed MOM38 or Isp42), Tom6 (Isp6), Tom7 (MOM7), and Tom5 (MOM8) (7–9, 11).

The other components of the TOM complex are partially exposed to the cytosol. Tom20 (MOM19/Mas20) and Tom22 (MOM22/Mas17) cooperate to form a presequence receptor at the mitochondrial surface and are thought to be the main entry point for preproteins into mitochondria (13). In keeping with the pivotal role of Tom20 and Tom22 in preprotein recognition, studies using deletion mutants showed that Tom20 is crucial for the biogenesis of mitochondria (14–16). Tom22 is essential for cell viability (17–19).

Another preprotein receptor, Tom70 (MOM72/Mas70), stimulates the import of a subset of preproteins (20–22), including the ADP/ATP carrier (AAC), the phosphate carrier, alcohol dehydrogenase isozyme III, and cytochrome c1 (23–25). In yeast, a subcomplex formed by Tom70 and Tom37 has been suggested to contribute to the efficiency of preprotein recognition (26). The Tom37-Tom70 subcomplex may form a docking point for preproteins such as preadrenodoxin (27), which becomes targeted to mitochondria through its interaction with the cytosolic protein MSF (mitochondrial import stimulation factor; Ref. 28). For other Tom37-Tom70-dependent preproteins such as AAC, a requirement for binding to MSF prior to their interaction with the mitochondrial surface has not been demonstrated.

From the Tom37-Tom70 receptor unit, preproteins are transferred to Tom20-Tom22 and enter the main import route (27, 29). In the absence of Tom70, preproteins can be directly recognized and imported via Tom20-Tom22, albeit without what reduced efficiencies (20). The Tom37-Tom70 receptor unit may, therefore, serve to increase the targeting efficiency of those preproteins that are recognized with lower affinity by Tom20-Tom22. This is in agreement with the observation that yeast cells lacking Tom70 (30) or Tom7 (26) grow slightly slower only on nonfermentable carbon sources at higher temperatures.

Recently, the sequencing of yeast chromosome VIII revealed an open reading frame, YHR117w, that encodes a Tom70-like protein (31), which we term Tom71. We investigated the expression, subcellular localization, and functional role of Tom71, and we assessed the possibility that the mild phenotypical consequences of deleting TOM70 were due to compensatory effects of TOM71.

EXPERIMENTAL PROCEDURES

Growth and Genetic Manipulation of Yeast Strains—Standard procedures were used for the growth of yeast cultures (32). Cells were grown on YP medium (1% (w/v) yeast extract and 2% (w/v) Bactopeptone) containing either 2% (w/v) glucose (YPD) or 3% glycerol and 2% (w/v) ethanol (YPGE). Strain S150 (MATα leu2–3, 112 his3–11 trpl1–289 ura3–52) was used previously to generate a disruption in TOM70 (S150tom70; Ref. 20). In the current study, the TOM71 gene was disrupted in both S150 and S150tom70 strains to generate the single and double mutants, S150tom71 and S150tom70tom71, respectively. The Δtom71 strain YSG1 (MATα ura3 leu2–3, 112 his4 lys2 mas37:His4) was a generous gift of Dr. S.D. Kohlwein (Universität Graz). Open reading frame YHR117w (EMBL/GenBank accession no. U00059) was amplified from genomic yeast DNA by PCR and cloned into pGEM4 (Promega) for in vitro transcription. The gene was dis-
ruptured by replacing an internal StuI restriction fragment (base pairs 170 to 350 of the coding sequence) with the HIS3 gene. Transformation of yeast cells with the resulting plasmid was carried out as described (33).

Miscellaneous Procedures—The following published or manufacturer’s procedures were used: standard DNA manipulations (34); coupling of peptides to ovalbumin with maleimide-activated carrier protein (Imject; Pierce), raising of antisera (35); blotting of proteins onto nitrocellulose, immunostaining with the ECL chemiluminescence detection system (Amersham); and in vitro transcription and translation reactions using [35S]methionine as a label (ICN Radiochemicals; Ref. 36). Mitochondria were isolated according to Daum et al. (37) and the postmitochondrial supernatant was retained for Tom71 localization experiments. In vitro import of radiolabeled preproteins, coimmunoprecipitation experiments, and chemical cross-linking of endogenous or imported proteins with 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide were performed as described (38).

RESULTS

A Gene for a Tom70-like Protein—Yeast chromosome VIII contains open reading frame YHR117w, which encodes a 639-residue protein (71,855 Da) with 53% amino acid identity and 70% similarity to Tom70 (Fig. 1; Refs. 31 and 39). This open reading frame was renamed TOM71, which follows the new rules for the nomenclature of the mitochondrial protein transport machinery (40) and avoids potential confusion with the former MOM72 (20). The strongest sequence similarity is found at the C termini of the proteins. The first small insert in Tom71 (amino acid residues 69–89) is positively charged and includes a potential nuclear localization signal (41). Tom71 shares 32 and 51% identity and chemical similarity, respectively, with Neurospora Tom70 (20).

The primary sequences of Tom71 and Tom70 share certain other features, including seven tetratricopeptide repeat motifs (Fig. 1; Ref. 20). Protein segments containing tetratricopeptide repeat motifs have been proposed to function in protein-protein interactions (42). Furthermore, a stretch of uncharged residues is present near the N termini of all three proteins (Fig. 1). This sequence anchors Tom70 to the mitochondrial outer membrane, exposing the large C-terminal domain to the cytosol (43). Within this sequence, both yeast proteins contain a series of alanines that have been proposed to lie on the same face of a membrane-embedded α-helix and form the surface responsible for the dimerization of Tom70 in the outer membrane (Ref. 44; Fig. 1, indicated by “A” above the alignment).

Tom71 Is Localized in the Outer Membrane of Yeast Mitochondria—To investigate the subcellular localization of Tom71, a polyclonal antibody was raised against a synthetic peptide corresponding to the C terminus of Tom71 (residues 628–639). This antibody recognized a single band with an apparent molecular mass of 66 kDa on Western blots of yeast mitochondria. This protein was absent in the postmitochondrial supernatant (Fig. 2A) and was not significantly enriched in nuclear fractions, suggesting that the stretch of positive charges does not function as a nuclear targeting signal (data not shown).

Tom71 was resistant to extraction with alkaline buffers (Fig. 2A), indicating that it is tightly associated with mitochondrial membranes. Upon treatment with trypsin, fragments of 57 and 51 kDa were released from the mitochondria and could be detected by immunostaining for the C terminus of Tom71 (residues 628–639). This antibody recognized a single band with an apparent molecular mass of 66 kDa on Western blots of yeast mitochondria. This protein was absent in the postmitochondrial supernatant (Fig. 2A) and was not significantly enriched in nuclear fractions, suggesting that the stretch of positive charges does not function as a nuclear targeting signal (data not shown). The “A” above the yTom70 sequence indicates the alanine residues thought to participate in dimerization of yTom70 in the mitochondrial outer membrane (44).
plex—To determine whether Tom71 is a part of the protein import complex of the outer membrane, radiolabeled Tom71 was imported into isolated mitochondria. Following lysis in digitonin, the TOM complexes were isolated by co-immunoprecipitation with various antibodies (Fig. 2C). Imported Tom71 could be precipitated with antisera against Tom70, Tom40, and Tom71, whereas antibodies derived from preimmune serum precipitated significantly less Tom71. This suggests that Tom71 is associated with the receptor complex. The relatively low specific recovery of Tom71 with anti-Tom40 antibodies is reminiscent of the weak co-immunoprecipitation of Tom70 with this antisem (7). Previous experiments indicated that Tom70 is not a permanent component of the import complex but rather

**FIG. 2. Subcellular localization of Tom71.** A, total cell lysates (Tot., 100 μg of protein), mitochondria (Mit., 40 μg), and postmitochondrial supernatants (PM Sup, 40 μg) were subjected to SDS-PAGE, followed by immunostaining for Tom71, Tom70, Tom20, and the cytoplasmic protein Ycr77p (Ref. 49; left panel). Mitochondria were extracted with 100 mM sodium carbonate (pH 11.5; Alk. extr., right panel). After centrifugation at 100,000 × g for 1 h at 2 °C, the membrane pellet (Pel) and the supernatant (Sup) fractions were precipitated with trichloroacetic acid, and proteins were analyzed by SDS-PAGE and immunostaining. CS indicates citrate synthase, a soluble protein of the mitochondrial matrix. B, mitochondria (100 μg) were treated with the indicated concentrations of trypsin at 0 °C for 15 min. Digestion was stopped by addition of a 20-fold (w/w) excess of soybean trypsin inhibitor, and the organelles were collected by centrifugation. The pellet (P) and supernatant (S) fractions were analyzed as described above. The two tryptic fragments arising from Tom71 are labeled F1 and F2, while F indicates the soluble fragments of Tom70. The intermembrane space protein cytochrome bɔ (Cyt bɔ) was not degraded during the tryptic treatment, indicating that the outer membrane had remained intact. C, co-immunoprecipitation of Tom71 with the protein import complexes. Radiolabeled Tom71 was imported into isolated mitochondria, as described for Tom70 (38). The organelles were then collected by centrifugation, and dissolved in buffer containing 0.5% digitonin as described (7). The TOM complexes were isolated by co-immunoprecipitation in the presence of 125 mM NaCl, using the indicated antibodies. As a negative control, antibodies derived from preimmune serum (PIS) were used. Immunoprecipitated Tom71 was analyzed by SDS-PAGE and autoradiography and quantitated by densitometry. The results are presented relative to the amount of Tom71 precipitated with the Tom71-specific antiserum.
is associated with it in a dynamic fashion (22, 21). Our co-immunoprecipitation data suggest that the same is true for Tom71.

The association of Tom71 with other proteins of the outer membrane was assessed in cross-linking experiments with mitochondria containing radiolabeled, imported Tom71. A radiolabeled product with an apparent molecular mass of about 100 kDa was observed after cross-linking with the reagent 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide, demonstrating a dose association of Tom71 with a 30–35-kDa protein (Fig. 3). This cross-linked species comigrated in SDS-PAGE with a product generated in a parallel experiment using imported Tom70 (Fig. 3; Ref. 38). The intensity of the Tom71/cross-link product was increased in mitochondria lacking Tom70, suggesting that Tom71 and Tom70 interact with the same protein in the outer membrane. The most likely candidate for the cross-link partner was Tom37, which has been reported to form a stable complex with Tom70 (26). However, the cross-link product was observed at wild-type efficiency after import of either Tom71 or Tom70 into mitochondria isolated from a Tom73 deletion mutant (Fig. 3). Another potential candidate was Tom40, a component of the general insertion pore (7, 11). Several antibodies raised against Tom40 did not recognize the cross-link product. Therefore, the cross-linking partner remains to be identified.

Tom71 Is a Nonessential Protein, Even in the Absence of Tom70—Yeast strains harboring single or double disruptions of the TOM71 and TOM70 genes were generated to investigate the potential role of Tom71 in mitochondrial biogenesis. The disruptions were confirmed by immunostaining analysis of mitochondria isolated from the mutant cells (Fig. 4A). Growth of the resulting strains, S150Δtom71, S150Δtom70 and S150Δtom70Δtom71, was analyzed on both fermentable and nonfermentable carbon sources. The growth phenotypes of the single and double mutants were indistinguishable from that of the wild-type strain on glycerol-containing medium at 24°C (data not shown) and 30°C (Fig. 4B). At 37°C, however, the single and double deletion strains showed reduced growth, relative to wild-type cells. Both strains lacking Tom70 grew at similar rates, which were slightly lower than that of S150Δtom70, suggesting that the slow growth of the double mutant is due to the lack of Tom71. Reduced growth was caused by impaired mitochondrial function at the elevated temperature, since the mutant cells grew at wild-type rates on glucose-containing medium under these conditions (data not shown).

The protein composition of mitochondria isolated from the set of yeast mutants was not significantly altered (data not shown). In particular, the levels of Tom70 remained unchanged in the Δtom71 mitochondria, as did those of Tom20, porin, and the intermembrane space protein cytochrome c1 (CC1HL; Fig. 4A). A similar result was seen in mitochondria lacking Tom70. The amounts of TOM70 or TOM71 mRNA were also unchanged in the respective single deletion strains (data not shown). Thus, the insertion and maintenance of either Tom71 or Tom70 in the outer membrane does not depend on the second protein. Furthermore, the level of one component does not respond to the presence or absence of the other.

Protein Import Is Not Significantly Reduced in Cells Lacking Tom71—The involvement of Tom71 in mitochondrial protein import was tested by measuring the surface binding and import of various preproteins. As demonstrated previously (20), the binding of AAC is significantly reduced in mitochondria lacking Tom70. With mitochondria from the TOM71-deletion strain, a less pronounced but significant decrease in binding was observed (Fig. 5A). In the double mutant lacking Tom70 and
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Tom71, binding was reduced to the background level seen with trypsin-pretreated mitochondria, from which surface receptors had been degraded. Thus, AAC seems to be able to bind to Tom71, albeit with lower affinity than to Tom70. Similar results were obtained for the import of AAC into the various mitochondria. The reduced import efficiencies were reflected in the steady-state levels of AAC in isolated mitochondria (Fig. 4A). Therefore, Tom71 appears to make a small contribution to the surface binding and import of AAC. Likewise, import into the Δtom71 mitochondria of two other preproteins known to use the Tom70 receptor, namely the phosphate carrier (23), and precytochrome c (22, 25) was not significantly reduced (Fig. 5B).

The import of a number of precursors that utilize the Tom20-Tom22 receptor unit was analyzed. These included preproteins destined for each of the four mitochondrial subcompartments that either lacked (porin, cytochrome c, heme lystate, or BCS1) or contained (α-subunit of the matrix-processing peptidase, Su9-DHFR, or F$_{1}$-ATPase β-subunit) N-terminal, degradable presequences. In almost all cases, no significant changes in the import into the mutant mitochondria was detected in comparison to that into wild-type organelles (Fig. 5B). The reduction by 40% of porin import into the double mutant mitochondria was unexpected, because previous studies have shown that this preprotein is imported via Tom20-Tom22 (29, 35). The import of the β-subunit of the F$_{1}$-ATPase (Fig. 5B, F$_{1}$β) was enhanced by 60% in the double-disrupted mitochondria. The reason for these variations is not known. In summary, Tom71 is not required for the efficient import of any of the precursor proteins tested but may participate to some extent in the specific binding and import of AAC.

**DISCUSSION**

Tom71 is a novel homologue of Tom70, a preprotein receptor of the mitochondrial outer membrane. The two proteins are strikingly similar in terms of primary sequence, structural arrangement in the membrane, association with the TOM complex, and proximity to a protein of 30–35 kDa. In spite of these compelling similarities, our studies have demonstrated that Tom71 is not required for the import of preproteins known to use Tom70, or in fact, any of the precursors that were examined. Given the large number of mitochondrial proteins whose import requirements have not been determined, it cannot be ruled out that a subset of precursors uses Tom71, either alone or in combination with Tom70, as the primary import receptor. This model could explain the more severe growth defect observed in cells lacking Tom71, in comparison to the Tom70 null mutants.

Deletion of both TOM70 and TOM71 did not exacerbate the minor growth defects of single null mutant cells, suggesting that Tom71 does not compensate for the absence of its homologue, Tom70. Therefore, Tom71 and Tom70 together do not appear to perform any functions that are essential for mitochondrial biogenesis.

Our cross-linking studies place Tom70 and Tom71 in the vicinity of a 30–35 kDa protein. Experiments utilizing Δtom71 mutant mitochondria clearly demonstrate that the cross-link partner is not Tom73, raising the possibility that the Tom complex contains another yet to be identified component. A recently published series of experiments suggests that yeast Tom70 and Tom73 can interact to form a docking site for the presequence-containing preprotein preadrenodoxin from rat liver (27, 28). This protein is thought to be targeted to the organelles in an ATP-dependent step by the MSF (45). Our import studies suggest that not all preproteins that require cytosolic ATP for import have to enter mitochondria via the Tom73-Tom70-Tom71 receptors. For instance, the import of the β-subunit of the F$_{1}$-ATPase and of the α-subunit of the matrix-processing peptidase was not affected in Δtom70 and Δtom71 mutant mitochondria, although these precursors require ATP for import (46). Similarly, the import of porin strongly depends on ATP (47), and porin preprotein stimulates the ATPase activity of MSF (28). Nonetheless, porin import was hardly affected in the mutant mitochondria (Fig. 5; Ref. 20), showing that MSF-Tom37-Tom70-mediated docking to the mitochondrial surface is not an essential step.

What alternative functions could be performed by Tom71? Our studies render it unlikely that Tom71 is involved in the final assembly or stability of the TOM complex. An interesting possibility is that Tom71 mediates interactions between mitochondria and other cellular proteins or the cytoskeleton, as was originally suggested for Tom70 (20). In this respect, it is noteworthy that Tom71, like Tom70, contains seven tetratricopeptide repeat motifs. Sequences harboring these motifs mediate protein-protein interactions (42, 48), but at present it is not known what function these motifs perform in the mitochondrial proteins. We did not find evidence for Tom71 in the nucleus, despite the presence of a potential nuclear localization signal. However, the presence of a minor fraction (5% or less) of total Tom71 in the nuclei cannot be excluded. In addition, certain conditions could lead to targeting of Tom71 to the nucleus. In conclusion, further investigation is required to elucidate the intracellular function of Tom71.

Tom71 and Tom70 are the first set of highly homologous components of the TOM complex to be identified. TOM71 (chromosome VII) and TOM70 (chromosome I) are only one pair of many similar genes that are reiterated on different chromosomes (31). These observations raise the interesting possibility that other components of the import machinery such as Tom37 are duplicated. Clearly, the existence of highly homologous and functionally overlapping proteins requires careful interpretations of the import characteristics of mutant mitochondria.

Acknowledgments—The expert technical assistance of A. Weinzierl and P. Heckmeyer and the contributions of M. Dembowski are appreciated. We thank Dr. S. D. Kohlwein (Graz) for providing the Δtom71 yeast strain and Dr. E. Schiebel (Munster) for antibodies against the yeast nuclear protein Nu1p.

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