Role of Tim21 in Mitochondrial Translocation Contact Sites*

Received for publication, March 24, 2005, and in revised form, May 3, 2005
Published, JBC Papers in Press, May 4, 2005
DOI 10.1074/jbc.C500135200

Dejana Mokranjac‡, Dušan Popov-Čeleketić‡, Kai Hell, and Walter Neupert§
From the Institut für Physiologische Chemie, Universität München, Butenandtstrasse 5, 81377 München, Germany

Translocation of preproteins with N-terminal presequences into mitochondria requires the cooperation of the translocase of the outer membrane (TOM complex) and the presequence translocase of the inner membrane (TIM23 complex). However, the molecular nature of the translocation contact sites is poorly understood. We have identified a novel component of the TIM23 translocase, Tim21, which is involved in their formation. Tim21 is anchored in the mitochondrial inner membrane by a single transmembrane domain and exposes its C-terminal domain into the intermembrane space. The purified C-terminal domain of Tim21 appears not to bind to any of the TIM23 components but rather specifically interacts with the TOM complex. We propose that Tim21 binds to the trans site of the TOM complex thus keeping the two translocases in close contact.

Import of preproteins with matrix-targeting signals (presequences) from the cytosol into mitochondria requires the concerted action of the translocase of the outer membrane (TOM complex) and the preprotein translocase of the inner membrane (TIM23 complex) (for reviews on protein import into mitochondria, see Refs. 1–4). In the presence of an arrested translocation intermediate the TOM and the TIM23 complexes could be co-isolated (5, 6). However, a direct interaction between components of the TOM and the TIM23 complexes has not been observed so far. The TOM complex consists of receptor subunits and the general insertion pore made up from the central pore-forming component Tom40, the receptor Tom22, and small Tom subunits. Following transport of presequences across the outer membrane via the pore of the TOM complex the preproteins are bound at the trans site of the TOM complex. The molecular nature of the trans site is not exactly clear, but segments of Tom40, the IMS domain of Tom22, and Tom7 were reported to be involved in binding of preproteins at the trans side of the membrane (7–10). The preproteins are then transferred to the TIM23 complex, which can be divided into a tightly membrane-integrated part with hydrophilic domains in the intermembrane space and the import motor. Two components of the membrane-integrated part, Tim23 and Tim17, are believed to form the preprotein-conducting channel (5, 11, 12), whereas Tim50 appears to exert its function in the transfer of preproteins from the TOM to the TIM23 translocase (13–15). The N-terminal hydrophilic domain of Tim23 has a role in tethering the inner membrane translocase to the outer membrane bringing both membranes in close contact (16). This appears to facilitate the preprotein transfer between the translocases and to thereby increase the efficiency of protein import.

The import motor, made up from five known proteins, Tim14, Tim16, Tim44, Mge1, and mtHsp70, is attached to the membrane part at the matrix face of the inner membrane. It completes translocation of the preprotein across the inner membrane by ATP-driven cycles of binding to and releasing from mtHsp70, leading to the vectorial movement into the matrix.

We report here the identification of a novel component of the TIM23 translocase, Tim21. It is anchored in the mitochondrial inner membrane and exposes its C-terminal domain into the IMS. The C-terminal domain of Tim21 specifically interacts with the TOM complex. We propose that Tim21 is involved in the formation and stabilization of the mitochondrial translocation contact sites.

EXPERIMENTAL PROCEDURES

Plasmids, Yeast Strains, and Cell Growth—Wild type yeast strain YPH499 was used (17). Protein A-tagged Tim23 was made by amplifying the two IgG-binding domains of Protein A by PCR using pYM9 (18) as a template and cloning it between the TIM23 promoter and reading frame in the pGEM4 vector. The whole construct was subcloned into the pSS515 yeast vector (17). This plasmid was transformed into a haploid deletion strain of YPH499 leading to the ‘‘TOM23 HA’’ strain. Yeast cells were normally grown in lactate medium containing 0.1% glucose.

The nucleotide sequence coding for the intermembrane space domain of Tim21 (Tim21IMS) was amplified using primers BamTim21_97Duet (5′-CCC GGA TCC GTC AGA ACT ATT TTC GCC TTC AG-3′) and Tim22Inh_new (5′-TTT AAG CTC ATT TTA ATT TCT TCT GGG GCCG-3′) and yeast genomic DNA as a template. The PCR fragment was cloned in the pETDuet vector (Novagen), and the protein, containing an N-terminal hexahistidine tag, was expressed in BL21(DE3) cells upon induction with isopropyl β-D-thiogalactopyranoside. Protein was purified under native conditions using a Ni-NTA agarose column (Qiagen) according to the manufacturer’s instructions.

Purification of the TOM23 Complex—Wild type mitochondria or mitochondria from a strain containing Protein A-tagged Tim23 were solubilized at 1 mg/ml with 1% digitonin (w/v) in 20 mM Tris/HCl, 80 mM KCl, pH 7.2, 1 mM phenylmethylsulfonyl fluoride at 4 °C. After a clarifying spin, mitochondria from a strain containing Protein A-tagged Tim23 were solubilized at 1 mg/ml with 1% digitonin (w/v) in 20 mM Tris/HCl, 80 mM KCl, 10 mM imidazole, pH 7.2, 1 mM phenylmethylsulfonyl fluoride). Wild type mitochondria were solubilized in binding buffer for 20 min at 4 °C. After a clarifying spin, the soluble fraction was immobilized His6-Tim21IMS was equilibrated in the binding buffer (1% digitonin (w/v) in 20 mM Tris/HCl, 80 mM KCl, 10 mM imidazole, pH 7.2, 1 mM phenylmethylsulfonyl fluoride). Wild type mitochondria were solubilized in binding buffer for 20 min at 4 °C. After a clarifying spin,
RESULTS AND DISCUSSION

Tim21, a Novel Component of the TIM23 Translocase—To purify the TIM23 complex from mitochondria of *S. cerevisiae*, we constructed a strain carrying a Protein A tag at the N terminus of Tim23. The strain grew like wild type yeast under all conditions tested. Mitochondria were isolated, lysed with digitonin, and the TIM23 complex was isolated by IgG-Sepharose affinity chromatography. Samples were analyzed by SDS-PAGE and immunodecoration. The total (T) and bound fractions (B) were analyzed by SDS-PAGE and immunodecoration. The total (Total) contains 20% of the material present in input. B, purified preprotein, pb2(167)ΔDHFR-His6, was arrested in the presence of methotrexate as an intermediate spanning both TOM and TIM23 complexes using mitochondria which contained Tim21 with an HA tag. After lysis with digitonin, solubilized material was incubated with Ni-NTA-agarose chromatography. Samples were analyzed by SDS-PAGE and immunodecoration. The total (Total) contains 10% of the material present in input.

Solubilized material was incubated for 1 h with the Ni-NTA-agarose beads. After three washing steps with the binding buffer, bound proteins were eluted with Laemmli buffer containing 300 mM imidazole. Total and supernatant fractions contain 20% of the material present in input. B, purified preprotein, pb2(167)ΔDHFR-His6, was arrested in the presence of methotrexate as an intermediate spanning both TOM and TIM23 complexes using mitochondria which contained Tim21 with an HA tag. After lysis with digitonin, solubilized material was incubated with Ni-NTA-agarose beads. In parallel, a reaction was performed in the absence of the preprotein. After brief washing steps, bound proteins were eluted with Laemmli buffer containing 300 mM imidazole. Total (T) and bound fractions (B) were analyzed by SDS-PAGE and immunodecoration. The total (Total) contains 10% of the material present in input.

the eukaryotic kingdom. A region with particularly high conservation is present in the predicted transmembrane domain and to a lesser extent in the C-terminal domain (data not shown).

We generated a strain with an HA tag at the C terminus of Tim21. Treatment of mitochondria with proteinase K left the HA-tagged Tim21 intact, in contrast to the outer membrane protein Tom70, which was completely degraded (Fig. 1C). Tom71 was accessible to the added protease only after opening of the outer membrane by hypotonic swelling, like the intermembrane space protein cytochrome *b5*. After treatment of mitochondria with high pH, Tim21 was recovered in the membrane fraction along with the membrane protein Tom70. To characterize the association of Tim21 with the TIM23 complex, mitochondria that contain HA-tagged Tim21 were
Is Tim21 also part of the active translocase? To address this question we analyzed the association of Tim21 with the TOM and TIM23 translocases containing accumulated translocation intermediate. For this purpose, a mitochondrial precursor protein, cytochrome b$_2$ (167)$_2$DHFR, was incubated with mitochondria in the presence of methotrexate to generate a two-membrane spanning intermediate (5, 13). Mitochondria were solubilized with digitonin and incubated with Ni-NTA-agarose beads to isolate the TOM-TIM23 supercomplex via the His tag on the arrested precursor. Tim21 is part of this supercomplex as it was specifically bound to the Ni-NTA-agarose beads in the presence of the translocation intermediate (Fig. 2B). All other subunits of the TOM and TIM23 complexes analyzed were also found in this supercomplex. In contrast, AAC, one of the most abundant proteins of the inner membrane, was absent, confirming the specificity of the interaction. We observed copurification of Tim21 only with the precursor that was arrested across both membranes. No copurification was observed with precursor that was translocated only across the outer membrane or with precursor which was fully imported into the matrix (data not shown). Also, Tim21 did not associate with mitochondria after solubilization in digitonin (data not shown).

Next we analyzed the importance of Tim21 for the function of the TIM23 translocase. A null mutant of Tim21, constructed in the high-throughput deletion study, is viable (21). The deletion strain made in our laboratory displayed no obvious growth defect on either fermentable or nonfermentable carbon sources (data not shown). This is in contrast to all other known components of this complex that are essential for cell viability. We were also not able to detect any major defect in the ability of isolated mitochondria that lack Tim21 to import precursors that use the TIM23 translocase (data not shown). Thus, Tim21 does not appear to be crucial for the translocation of preproteins via the TIM23 complex. Rather, these results would suggest a regulatory or a stabilizing role of Tim21 within the TIM23 translocase. This is similar to the proposed roles of nonessential components of other translocases such as Tim18 in the TIM22 translocase or Tom6 and Tom7 in the TOM complex (22, 23).

The IMS Domain of Tim21 Interacts with the TOM Complex—To address the role of Tim21 within the TIM23 translocase we set out to identify binding partners of the intermembrane space domain of Tim21. We expressed the C-terminal domain of Tim21 with a N-terminal His$_6$ tag in Escherichia coli cells and purified it to homogeneity (Fig. 3A). Mitochondria were lysed with digitonin and incubated with Ni-NTA-agarose beads with or without Tim21$_{IMS}$ immobilized on it. Surprisingly none of the components of the TIM23 complex was specifically enriched in the bound fraction. Instead, the components of the TOM complex, Tom40 and Tom22, were specifically bound to the intermembrane space domain of Tim21. As Tim21 is clearly a component of the TIM23 translocase and not of the TOM complex, it appears that the interaction of Tim21 with the components of the TIM23 complex is mediated by its transmembrane domain. This in agreement with the higher degree of sequence conservation of this domain as compared with the sequence similarity of the IMS domain.

We suggest that the intermembrane space domain of Tim21 interacts with the trans binding site of the TOM complex and thereby stabilizes the interaction of the two translocases, together with the N-terminal domain of Tim23 and probably Tim50. The contribution of the latter two components to TOM-TIM23 contacts would explain why deletion of Tim21 does not have a strong inhibitory effect on the rate of protein import (13–16).

Acknowledgments—We are grateful to Heiko Germeroth, Ulrike Gärtner, and Marica Malesic for excellent technical assistance and to Axel Imhof and Tilman Schlunk for mass spectrometry analysis.

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