Association of the Tim14·Tim16 Subcomplex with the TIM23 Translocase Is Crucial for Function of the Mitochondrial Protein Import Motor*

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Tim14 and Tim16 are essential components of the import motor of the mitochondrial TIM23 preprotein translocase. Tim14 contains a J domain in the matrix space that is anchored in the inner membrane by a transmembrane segment. Tim16 is a J-related protein with a moderately hydrophobic segment at its N terminus. The J and J-like domains function in the regulation of the import motor. Besides its recruiting function for mtHsp70, Tim44, Tim14 (Pam18), Tim16 (Pam16), and Mge1 (18–28). With the help of these subunits mtHsp70 converts the energy of ATP hydrolysis into vectorial movement of the unfolded polypeptide chain across the translocation pore. MtHsp70 is part of the highly conserved Hsp70 family whose members bind to unfolded polypeptide chains in a reversible, ATP-dependent manner (29, 30). In the ATP state, the peptide binding domains of Hsp70s have an open conformation and they bind polypeptide chains with low affinity and high exchange rates. ATP hydrolysis by the nucleotide binding domain of Hsp70 triggers a conformational change of the peptide binding domain to its closed state so that this domain tightly binds to the polypeptide. The polypeptide is released upon dissociation of ADP and rebinding of ATP. This nucleotide exchange reaction is catalyzed by members of the GrpE family, in the case of the import motor the soluble matrix protein Mge1.

MtHsp70 is recruited to the translocation pore of the TIM23 translocase by Tim44, a protein associated with the inner membrane (31–33). Thus, it can immediately bind to the incoming unfolded polypeptide chain when it emerges from the translocation pore. MtHsp70 prevents backsliding of the polypeptide chain. To allow vectorial movement of the incoming polypeptide chain into the matrix space, bound mtHsp70 has to be released from Tim44 during the ATP-driven reaction cycle of the import motor.

Tim44 can be regarded as a scaffold for the organization of the import motor. Besides its recruiting function for mtHsp70, it is required for the association of two further subunits of the import motor, Tim14 and Tim16, to the TIM23 translocase (21). Tim14 belongs to the class of J domain co-chaperones that stimulate the ATPase activity of Hsp70 chaperones and thereby...
promote tight substrate binding by Hsp70s (21–23). Tim14 has one transmembrane segment that anchors the protein to the inner membrane with the J domain facing the matrix space. Association of Tim14 with the TIM23 translocase needs Tim16, which forms a stable subcomplex with Tim14 (24, 25). Tim16 has two conserved domains, the N terminus containing a segment of hydrophobic amino acid residues and a J-like domain. The J-like domain lacks the canonical HPD motif of J domain co-chaperones that is required for the stimulation of the ATPase activity of Hsp70. Indeed, Tim16 has no J protein activity but, rather, inhibits the stimulation of the ATPase activity by Tim14 in vitro (34–37). It is under discussion whether Tim16 modulates the stimulatory effect of Tim14 on mtHsp70 in vivo. Formation of the Tim14-Tim16 subcomplex requires the conserved matrix part of Tim14 including the J domain and the conserved J-like domain of Tim16 (34, 37).

Tim16 forms also a complex with Mdj2, a close homolog of the J protein Tim14 (36). Mdj2 can be present in the import motor instead of Tim14. It appears to function in a manner similar to Tim14, because it is able to rescue the deletion of Tim14 upon overexpression. In wild type cells it is present in lower amounts compared with Tim14. It is not required for viability of yeast cells, and its function is not clear to date. Pam17 is another non-essential protein whose deletion affects the import of presequence proteins into the mitochondrial matrix (38).

It is still under debate how the motor functions. To understand its molecular mechanism, a detailed functional analysis of the motor components and its domains is needed. Here we report on the roles of the characteristic N-terminal segments of Tim14 and Tim16. Although their J and J-like domains have been studied in detail, the roles of these segments have not been investigated. We show that the transmembrane segment of Tim14 and the conserved hydrophobic N terminus of Tim16 are not required for the formation of the Tim14-Tim16 subcomplex but rather play an important role in the association of the Tim14-Tim16 complex with the TIM23 translocase. The absence of both hydrophobic segments in Tim14 and Tim16 is lethal for yeast cells. We conclude that association of the Tim14-Tim16 subcomplex with the membrane-embedded part of the TIM23 complex is crucial for the import of preproteins by the TIM23 complex.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Yeast Strains, and Cell Growth**—The TIM14 open reading frame together with its promoter (400 base pairs upstream) and its terminator region (402 base pairs downstream) and the TIM16 open reading frame together with its promoter (327 base pairs upstream) and its terminator region (410 base pairs downstream) were cloned with the restriction sites SacI and XhoI into the vector pGEM4. In the case of the Tim14Δ60 variant the promoter region (restriction sites SacI and KpnI) and the nucleotide sequence coding for the start methionine and the amino acid residues 1 to 69 of Tim14 plus the terminator region (BamHI and XhoI) were cloned as two fragments in pGEM4. For the variants with the mitochondrial presequence of the subunit 9 of the F$_1$F$_O$-ATPase from *Neurospora crassa* the nucleotide sequence coding for amino acid residues 1 to 69 of subunit 9 was cloned in pGEM4 in front of the nucleotide sequence coding for amino acid residues 99 to 168 of Tim14 and amino acid residues 25 to 149 of Tim16, using the restriction sites BamHI and XbaI. Then the subcloned nucleotide sequences were transferred as SacI/XhoI fragment from pGEM4 to pRS314 plasmid, generating the plasmids pRS314-Tim14Δ60, pRS314-Su9Tim14Δ98, and pRS314-Su9Tim16Δ24.

The plasmids containing the TIM14 and TIM16 constructs were transformed into the respective haploid chromosomal deletion strains of TIM14 and TIM16 expressing the corresponding wild type genes from an URA3-carrying plasmid (36). Cells that had lost the URA3 plasmid expressing the wild type protein were selected on plates containing 5-fluoroorotic acid. The selected cells contained the pRS314 plasmid expressing the wild type or the variants of Tim14 and Tim16. Growth of these cells was tested on yeast extract-peptone dextrose (YPD) plates. Mitochondria were isolated from yeast cells grown at 30°C in YPD medium or lactate medium.

To test the growth of yeast cells in the absence of both hydrophobic segments of the TIM14-TIM16 complex a double deletion mutant of TIM16 and TIM14 harboring pRS314-Su9Tim16Δ24 and the TIM14 gene on a URA3-containing plasmid was generated. To this end, the chromosomal TIM14 open reading frame was replaced with the *KanMX4* cassette by homologous recombination in the TIM16 deletion strain harboring pRS314-Su9Tim16Δ24 and the TIM14 gene on a URA3-containing plasmid (40). Positive clones were selected on minimal medium plates lacking uracil and containing G418. The nucleotide sequences to express wild type Tim14 and the Su9Tim16Δ98 variant were subcloned from the plasmid pRS314 into the plasmid pRS315 containing the *Leu* auxotrophy marker. These plasmids were transformed in the generated double deletion strain. Viability of a strain harboring only the Tim16Δ24 and the Tim14Δ98 variants was tested by selection of cells that have lost the *URA* plasmid expressing wild type Tim14 on plates containing 5-fluoroorotic acid.

**Import of Proteins into Isolated Mitochondria**—Precursor proteins were synthesized in a standard or in a transcription and translation-coupled reticulocyte lysate system (Promega) in the presence of [35S]methionine. The import of precursor proteins into isolated mitochondria (0.5 mg protein ml$^{-1}$) was performed in import buffer (0.1% bovine serum albumin (w/v), 500 mM sorbitol, 50 mM HEPES-KOH, 80 mM KCl, 10 mM MgAc$_2$, 2.5 mM EDTA, 2 mM potassium phosphate, and 1 mM MnCl$_2$, pH 7.2) containing 4 mM NADH, 2 mM ATP, 10 mM creatine phosphate, 100 μg/ml creatine kinase. Following preincubation for 3 min at 25 or 12°C, the import reaction was started by addition of 1–3% reticulocyte lysate (v/v) containing the radiolaabeled precursor protein and further incubated. At indicated times aliquots were withdrawn. Aliquots were divided and diluted either in SH buffer (600 mM sorbitol, 20 mM HEPES-KOH, pH 7.2) or SH buffer plus 50 μg/ml protease K and incubated for 20 min on ice. Protease treatment was stopped by addition of 2 mM phenylmethylsulfonyl fluoride.
Mitochondria were reisolated and washed and the import reaction was analyzed by SDS-PAGE, transfer to nitrocellulose membrane, and autoradiography. The amount of proteinase K-protected preprotein was quantified by laser densitometry (Pharmacia).

**Chemical Cross-linking**—Chemical cross-linking was performed in isolated mitochondria depleted of ATP. To deplete mitochondrial ATP mitochondria were treated with apyrase (10 units/ml) and oligomycin (10 μM) for 10 min at 25 °C in import buffer lacking bovine serum albumin. Disuccinimidyl glutarate was added to a final concentration of 0.15 mM and incubated for 30 min on ice. The cross-linking reaction was stopped by addition of 0.1 m glycine, pH 8.8. Mitochondria were reisolated and mitochondrial proteins were analyzed by SDS-PAGE and immunodecoration.

**Miscellaneous**—Sonication experiments were performed according to Rowley et al. (41). Mitochondria were diluted in buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride and sonicated in the presence of various concentrations of KCl. Separation into membrane and supernatant fraction was controlled by immunodecoration with antibodies against the marker proteins ADP/ATP-Carrier (AAC) and Mge1. Coimmunoprecipitation experiments were performed as previously described (36).

**RESULTS**

**Cells Containing a Tim14 Variant That Lacks the Transmembrane Segment Are Compromised in Growth**—Tim14 consists of an N-terminal intermembrane space (IMS) domain, a hydrophobic transmembrane (TM) segment, and a positively charged amphipathic helix followed by a highly conserved part including the J domain (Fig. 1A). To determine the function of the IMS domain and the membrane anchor we generated N-terminal truncation mutants. The Tim14Δ60 mutant lacks the N-terminal IMS domain of Tim14. This domain is not required for targeting of Tim14 to mitochondria. It is the transmembrane segment together with the following amphipathic helix that apparently forms an internal mitochondrial targeting signal. To target the conserved C-terminal part of Tim14 (Tim14Δ98) to the matrix space it was fused to the precursor of the subunit 9 of the F1Fo-ATPase. Upon import of the fusion protein pSu9Tim14Δ98 into mitochondria the precursor was proteolytically removed.
Yeast strains were constructed that express these truncated forms instead of wild type Tim14 (37). Drop dilution tests were performed on plates containing rich glucose medium (YPD). Deletion of the N-terminal intermembrane space tail of Tim14 did not affect growth (Fig. 1B). Cells expressing Tim14 without the TM segment showed reduced growth rates, in particular at the higher temperature of 37 °C. Thus, the TM segment of Tim14 is important but not essential for the function of Tim14, whereas its N-terminal IMS domain plays no or only a minor role.

Import of Matrix Proteins into Mitochondria Is Affected in Tim14 Mutant Lacking the Transmembrane Segment—Because Tim14 functions in the mitochondrial import motor of the TIM23 translocase, we tested whether the observed growth defects correlate with defects in the import of substrates of the TIM23 translocase. The pre-subunit β of F1-ATPase showed decreased import rates into isolated mitochondria containing Tim14Δ98 as compared with mitochondria harboring wild type Tim14 but similar import rates with mitochondria containing Tim14Δ60 (Fig. 2A). The same was observed for the inner membrane preprotein CoxVa, which requires a functional import motor (Fig. 2B). In contrast, import rates of CoxVa-(Δ26–89) whose transport is independent of the import motor were not significantly altered in mitochondria from both mutants (Fig. 2C). Normal import rates were also observed for the precursor of Tim23, a substrate of the TIM22 translocation pathway (Fig. 2D). Therefore, the import defects observed are not due to secondary effects such as lowered membrane potential. In summary, the translocation of preproteins that depend on the import motor is specifically affected in the Tim14Δ98 mutant. The import defects correspond to the reported growth phenotype.

The TM Segment of Tim14 Stabilizes the Interaction with the TIM23 Complex—We characterized the interactions of the mutant proteins with the inner membrane and the TIM23 complex. First, salt extractions of mitochondrial membranes were performed. Mitochondria isolated from the different Tim14 strains were sonicated in the presence of increasing concentrations of KCl. The samples were separated into membrane and supernatant fraction and analyzed by SDS-PAGE and immunoblotting with antibodies against Tim14 (Fig. 3A). Wild type Tim14, as well as Tim14Δ60, were found in the membrane fraction at all KCl concentrations tested (Fig. 3A). The Tim14Δ98 mutant protein was associated with the membrane at KCl concentrations lower than 250 mM. An increasing fraction of the protein was released to the supernatant fraction at higher salt. This shows that Tim14 is attached to the inner membrane even in the absence of the TM segment. However, this association is sensitive to high ionic strength.

To analyze the association of Tim14 variants with the TIM23 translocase, mitochondria were lysed in digitonin-containing buffer and coimmunoprecipitations were performed using immunodecoration with antibodies against Tim14 (Fig. 3A). Wild type Tim14, as well as Tim14Δ60, were found in the membrane fraction at all KCl concentrations tested (Fig. 3A). The Tim14Δ98 mutant protein was associated with the membrane at KCl concentrations lower than 250 mM. An increasing fraction of the protein was released to the supernatant fraction at higher salt. This shows that Tim14 is attached to the inner membrane even in the absence of the TM segment. However, this association is sensitive to high ionic strength.

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antibodies against the integral membrane component Tim17 and the motor component Tim16. Antibodies against Tim17 depleted Tim17 and Tim23 from mitochondrial extracts of cells harboring wild type Tim14 or Tim14Δ98 (Fig. 3B). In addition, from these extracts a portion of Tim44 and to a lesser extent Tim14 and Tim16 were isolated together with Tim17. Antibodies against Tim16 depleted Tim14Δ98 and Tim16 from the mitochondrial extracts and also precipitated part of Tim44, Tim23, and Tim17. Neither the interaction with Tim16 nor the interaction with the integral Tim23-Tim17 core complex and Tim44 were affected by the lack of the IMS domain of Tim14. Apparently, this domain does not play a role in the stability of the TIM23 translocase. In the Tim14Δ98 mutant, on the other hand, antibodies against Tim16 coprecipitated Tim14Δ98, but neither Tim44, nor Tim23 and Tim17 in significant amounts (Fig. 3B). This shows that the Tim14 and Tim16 subcomplex is stable in the absence of the first 98 amino acid residues of Tim14. In contrast, an interaction of the truncated Tim14 with Tim44 and the Tim23-Tim17 core complex was not detected. Antibodies against Tim17 pulled down Tim17 together with Tim23 and a part of Tim44. Only minor amounts of Tim16 and Tim14Δ98 were found in the precipitate. Part of Tim16 is associated with the TIM23 translocase in a subcomplex together with Mdj2, a non-essential homolog of Tim14 (36).

Interactions of low affinity might be missed when mitochondria are lysed even with very mild detergents. Therefore, we performed cross-linking experiments in intact mitochondria. As previously reported, Tim14 can be cross-linked to Tim44 and Tim16 (21, 24). Consistent with the results of the co-immunoprecipitation experiments, we observed cross-linked adducts of the Tim14 variants with Tim16, indicating the presence of intact Tim14-Tim16 subcomplexes (Fig. 3C, upper panel). Even with the Tim14Δ98 mutant mitochondria a cross-linked adduct of Tim14 protein to Tim44 was observed (lower panel). In this mutant Tim44 was also cross-linked to Tim16 (upper panel). This indicates that the Tim14Δ98-Tim16 complex is in close proximity to Tim44 in intact mitochondria. We conclude from the cross-linking experiments that the Tim14Δ98 mutant protein still interacts with the TIM23 translocase. However, this interaction is less stable than in the wild type. Together, the results suggest a stabilizing effect by the TM segment of Tim14 on the association of the TIM23 core with the Tim14-Tim16 subcomplex.

The N-terminal Part of Tim16 Is Important for Growth of Yeast Cells—The Tim14-Tim16 subcomplex contains another moderately hydrophobic segment in addition to the transmembrane segment of Tim14. This segment is present in the conserved N terminus of Tim16 and was predicted to contain a mitochondrial targeting signal, albeit with a relatively low score (42). We tested whether the N terminus is indeed able to function as targeting signal. To this end, we fused the first 29 amino acid residues in front of a passenger protein, mouse DHFR, to generate Tim16-(1–29)DHFR. This fusion protein could be imported into the mitochondrial matrix space. Its translocation required the presence of a mitochondrial membrane potential (Fig. 4B). We conclude that the first 29 amino acid residues of Tim16 have the potential to act as a mitochondrial targeting signal. Processing of the fusion protein was not observed. This is consistent with the lack of processing of the full-length Tim16 (24, 25).

The N terminus of Tim16, in contrast to matrix targeting signals, is highly conserved in terms of amino acid sequence. This suggested a role also in the function of the protein. We addressed this by expressing a version of Tim16 that lacked the first 24 amino acid residues. To allow targeting of the truncated protein to mitochondria the mitochondrial presequence from the subunit 9 of the F1F0-ATPase was fused to a Tim16 that lacked its N-terminal 24 amino acid residues (pSu9Tim16Δ24). Upon import into mitochondria the presequence of the fusion protein was proteolytically removed (Tim16Δ24). The growth rate of cells expressing Su9Tim16Δ24 was strongly reduced as compared with cells expressing wild type Tim16 (Fig. 4C). The N terminus of Tim16 obviously has another important function in addition to its role as a targeting signal.

Cells Expressing Tim16 Lacking the Hydrophobic N-terminal Segment Show Reduced Mitochondrial Import of Matrix Proteins—Preproteins that require the import motor of the TIM23 translocase showed strongly decreased import rates into mitochondria containing Tim16Δ24 compared with mitochondria containing wild type Tim16 (Fig. 5, A and B). On the other hand, minor import defects were observed for a motor-independent preprotein CoxVa that lacks the domain located in the matrix (CoxVa-(A26–89)) and for the ADP/ATP-Carrier ACC, a substrate of the TIM22 complex (Fig. 5, C and D). The results show that the hydrophobic N-terminal segment of Tim16 is crucial for the function of the import motor.
The N Terminus of Tim16 Is Crucial for Stable Association of the Tim16/Tim14 Subcomplex with the TIM23 Complex—To analyze the role of the hydrophobic segment of Tim16 we checked the association of Tim16 with the inner membrane and the TIM23 translocase. Upon salt extraction of mitochondrial membranes, up to 60% of Tim16 was released from the membrane fraction at 250 mM salt (Fig. 6A). In contrast, full-length Tim16 was observed mainly in the membrane fraction at all salt concentrations. We conclude that the N-terminal segment of Tim16 takes part in the membrane attachment of Tim16.

Coimmunoprecipitation experiments were performed to analyze interactions of the Tim16/Tim14 with components of the TIM23 translocase. The interaction with Tim14 in the Tim16-Tim14 subcomplex was not affected. However, neither Tim44 nor the Tim23-Tim17 core complex was co-isolated using antibodies against Tim16 from the extract of the Tim16 mutant (Fig. 6B). Consistent results were obtained with antibodies against Tim17. Truncated Tim16 and Tim14 could not be co-isolated with the Tim23-Tim17 core complex. Thus, the interaction of the Tim16-Tim14 subcomplex with the core complex is not stable in the absence of the hydrophobic segment of Tim16 upon detergent lysis.

Cross-linking experiments showed that Tim16Δ24 forms a stable complex with Tim14 (Fig. 6C, upper panel). A cross-linked adduct of Tim16Δ24 and Tim44 was detected that was slightly smaller in size than the Tim16-Tim44 cross-linked adduct observed in mitochondria containing wild type Tim16 (lower panel). In addition, Tim14 was cross-linked to Tim44 in mitochondria containing Tim16Δ24 (upper panel). Thus, at least portions of the subcomplex of Tim14 with truncated Tim16 are still in contact with Tim44. However, these interactions appear to be rather labile, as they are not preserved upon detergent lysis of mitochondria.

We conclude that the interaction of the Tim14-Tim16 subcomplex with the TIM23 core complex is severely destabilized in the absence of the hydrophobic N-terminal segment of Tim16. Although not absolutely essential for the interaction with the TIM23 translocase, this segment appears to play a very important role for the binding of Tim16-Tim14 subcomplex to the TIM23 translocase.

Finally, we analyzed the growth of cells lacking the hydrophobic segments in both proteins, Tim14 and Tim16. Tim14 and Tim16 were replaced by plasmid shuffling with Tim14Δ98 and Tim16Δ24 in the chromosomal deletion strain lacking TIM14 and TIM16. These cells did not grow at all (Fig. 6D). Deletion of both hydrophobic segments in Tim14 and Tim16 is therefore lethal although the truncated variants of Tim14 and Tim16 can form a complex (37). Most likely, the Tim14-Tim16 complex cannot associate with the TIM23 translocase in the absence of both segments.

In summary, the N-terminal hydrophobic segments are essential for the functional integration of the Tim14-Tim16 complex into the TIM23 complex and for the import of presequence-containing precursor proteins into the mitochondria.

FIGURE 5. The N-terminal segment of Tim16 is important for the import of preproteins dependent on the import motor. Mitochondria were isolated from strains harboring Tim16 or Tim16Δ24. Reticulocyte lysates containing the indicated radiolabeled precursor proteins were incubated with the mitochondria. The amounts of imported protein were analyzed as described in Fig. 2. A, pF1β, precursor to the β-subunit of ATP synthase; B, pCoxVa, precursor of subunit 5 of cytochrome oxidase; C, pCoxVa-(Δ26–89); D, AAC, ADP-ATP-Carrier.

DISCUSSION

In addition to their J and J-like domains that are responsible for the regulation of the activity of mtHsp70, Tim14 and Tim16 have characteristic hydrophobic segments in their N termini. In this report we have analyzed the function of these segments. Both segments have at least two functions. First, they function in the targeting of the proteins to the mitochondria and in their sorting to the inner membrane. Second, they play crucial roles in mediating the association with the TIM23 translocase.
Tim14 contains a TM segment followed by a stretch containing positively charged amino acid residues that has the potential to form an amphipathic helix. An import pathway for precursors containing this combination of targeting and sorting elements has been first described for the Bcs1 protein. These elements direct precursor proteins via the TIM23 translocase into the inner membrane with the N terminus facing the intermembrane space (43). Tim14 is not processed following import into mitochondria. A truncated form of Tim14 lacking the N-terminal IMS segment and the TM segment is only able to rescue the deletion of TIM14 when it is imported into mitochondria with a mitochondrial targeting signal (data not shown). Deletion of the N-terminal IMS segment alone does not affect the targeting of the protein. The TM segment apparently functions in translocation of Tim14 into mitochondria. Together with the subsequent amphipathic helix it most likely forms the mitochondrial targeting signal, such as suggested for the Bcs1 protein.

The N-terminal domain of Tim16 contains a moderately hydrophobic segment. Its amino acid sequence is conserved between yeast and human. It does not resemble a classical matrix-targeting signal. Furthermore, it is not cleaved off upon import (24, 25). The first 29 amino acid residues of Tim16 are sufficient to import the passenger protein DHFR into the mitochondrial matrix. Thus, Tim16 belongs to a class of proteins in the matrix space characterized by a conserved nonprocessed N-terminal import signal. How it interacts with the TOM (translocase of the outer membrane) and the TIM complexes remains to be elucidated.

The N-terminal segments of neither Tim14 nor Tim16 are required for the formation of the Tim14-Tim16 subcomplex in vivo. In the absence of the first 98 amino acid residues of Tim14 and the first 24 amino acid residues of Tim16 including the hydrophobic segments both proteins were still found in a subcomplex. Thus, the conserved matrix domain of Tim14 and the J-related domain of Tim16 are the conserved parts of the proteins sufficient to assemble the Tim14-Tim16 complex. This is consistent with earlier findings in which purified recombinant proteins comprising the conserved matrix part of Tim14 and the J-like domain of Tim16 formed a stable complex (35, 37). Furthermore, amino acid substitutions in the J domain of Tim14 (Pam18) destabilize the interaction between Tim14 and Tim16 in vivo (35).

The membrane segment of Tim14 and the N-terminal segment of Tim16 stabilize the association of the proteins with the inner membrane. With Tim14 stable association with the membrane was observed at even very high salt concentrations. The interaction between Tim14 and Tim16 in the Tim14-Tim16 subcomplex is not affected by the presence of
Functional Dissection of Tim14 and Tim16

high salt (37). Therefore, even in the absence of its moderately hydrophobic segment the part of Tim16 that is in complex with Tim14 is also membrane associated. Tim16 itself was largely found attached to the membrane. The N-terminal domain of Tim16 contributes significantly to this association.

The N-terminal segments of both Tim14 and Tim16 are crucial also for the interaction with the TIM23 core translocase. In particular, the N terminus of Tim16 appears to be important for the interaction. Still, the Tim16 Δ24 mutant strain was viable (37). An interaction of the Tim14-Tim16 subcomplex with the TIM23 translocase might not be essential for functional import or the interaction is more labile and thus not detectable after lysis of mitochondrial membranes. Because the Tim14-Tim16 complex can be cross-linked to Tim44 in intact mitochondria of the Tim14 and Tim16 mutants, the latter possibility is very likely. In addition, deletions of both hydrophobic segments in the Tim14-Tim16 subcomplex are lethal for yeast cells. These results support the hypothesis that mitochondrial import into the matrix space requires efficient and immediate trapping of the incoming precursor protein at the matrix side of the inner membrane by the associated import motor.

Tim44 most likely plays a crucial role in the interaction of Tim14 and Tim16 with the TIM23 complex and represents a potential binding partner for these two components. In its absence Tim14 and Tim16 are not assembled directly or at least only weakly with the TIM23 core complex (21). Pam17 has been reported to be a component of the import motor (38). Thus, it might be a candidate to mediate contact between the Tim14-Tim16 subcomplex and the other translocate components. However, Pam17 is not essential for viability of yeast cells, and its deletion does not abrogate the association of Tim14-Tim16 with the TIM23 core complex and Tim44.3 The purified N-terminal IMS domain of Tim14 (residues 2–65) expressed as glutathione S-transferase fusion protein was reported to bind specifically to Tim17 from mitochondrial extracts (44). On the other hand, our results indicate that cells expressing Tim14 without the IMS domain contain an intact TIM23 complex and have import rates not different from those of wild type cells. Thus, an interaction of the IMS domain of Tim14 with Tim17 appears not to be crucial for the assembly of the import motor to the TIM23 complex. This is in agreement with the lack of such N-terminal extensions in homologs of other fungi and higher eukaryotes.

In summary, the presence of at least one hydrophobic segment in the N termini of Tim16 and Tim14 is essential for the viability of yeast cells. The hydrophobic segments are crucial for the interaction of the Tim14-Tim16 subcomplex with the TIM23 translocase at the inner membrane. We conclude that the Tim14-Tim16 subcomplex is required to be present at the inner membrane, probably in close contact to the TIM23 translocase, to mediate efficient import of preproteins.

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