Organization and Function of the Small Tim Complexes Acting along the Import Pathway of Metabolite Carriers into Mammalian Mitochondria*

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Tim9, Tim10a, and Tim10b are members of the family of small Tim proteins located in the intermembrane space of mammalian mitochondria. In yeast, members of this family act along the TIM22 import pathway during import of metabolite carriers and other integral inner membrane proteins. Here, we show that the human small proteins form two distinct hetero-oligomeric complexes. A 70-kDa complex that contains Tim9 and Tim10a and a Tim9-10a-10b that is part of a higher molecular weight assembly of 450 kDa. This distribution among two complexes suggests Tim10b to be the functional homologue of yeast Tim12. Both human complexes are tightly associated with the inner membrane and, compared with yeast, soluble 70-kDa complexes appear to be completely absent in the intermembrane space. Thus, the function of soluble 70-kDa complexes as trans-site receptors for incoming carrier proteins is not conserved from lower to higher eukaryotes. During import, the small Tim complexes directly interact with human adenine nucleotide translocator (ANT) in transit in a metal-dependent manner. For insertion of carrier preproteins into the inner membrane, the human small Tim proteins directly interact with human Tim22, the putative insertion pore of the TIM22 translocase. However, in contrast to yeast, only a small fraction of Tim9-Tim10a-Tim10b complex is in a stable association with Tim22. We conclude that different mechanisms and specific requirements for import and insertion of mammalian carrier preproteins have evolved in higher eukaryotes.

Most mitochondrial proteins are encoded by nuclear genes, translated at cytosolic ribosomes, and imported to their final destination in the mitochondrion. Import of nuclear-encoded preproteins is a multistep process mediated by a common translocase in the outer membrane, the TOM1 complex, and two distinct translocases in the inner membrane, named TIM23 and TIM22 complex (1–4). The TOM complex is used by all known nuclear-encoded precursors and translocates them into and across the outer membrane. The TIM23 and the TIM22 complex show different substrate specificities and energy requirements. Presence-containing proteins are translocated across the inner membrane into the matrix via the TIM23 complex; whereas polytopic inner membrane proteins without presequences but with internal targeting signals use the TIM22 complex. The mitochondrial import pathways are evolutionary conserved and many components of the mitochondrial import machineries characterized in the fungal system have counterparts in mammalian mitochondria (5).

The TIM23 complex consists of the integral membrane components Tim23, Tim17, and Tim50. Tim23 and Tim17 constitute the protein-conducting channel and act in cooperation to form a receptor for presequences in the intermembrane space (6–8). Tim50, exposing its large C-terminal domain to the intermembrane space, was proposed to facilitate the transfer of precursor proteins to the translocation channel (9–11). Translocation across the inner membrane is initially driven by the membrane potential \( \Delta \Psi \), while further direction into the matrix is mediated by an ATP-dependent molecular motor at the matrix site of the inner membrane. It is composed of the peripheral membrane protein Tim44 that recruits the mitochondrial mHsp70 and the nucleotide exchange factor Mge1 (12–14). Recently, Tim14 was identified as a new component of the mitochondrial import motor that binds to Tim44 and mHsp70 in an ATP-dependent manner thereby activating mHsp70 (15).

Most hydrophobic proteins of the inner membrane such as the polytopic members of the mitochondrial carrier family are synthesized with internal targeting signals and inserted into the inner membrane via the TIM22 complex (16). In yeast, the TIM22 complex consists of the integral membrane components Tim22, Tim54, and Tim18. Tim22 is the major component of the TIM22 complex and is structurally related to Tim17 and Tim23 suggesting that both TIM proteins have evolved from a common ancestor (16). Similar to the TIM23 complex it forms a voltage-gated channel in the inner membrane (17). Tim54 exposes a large domain to the intermembrane space and is required for the stability of Tim22 but may not interact directly with translocating precursor proteins (18). Tim18 is structurally related to the subunit IV of the succinate dehydrogenase

branone; DDP1, deafness-dystonia peptide 1; \( \Delta \Psi \), membrane potential; PBS, phosphate-buffered saline; CCCP, carbonyl cyanide-p-trifluoro-

methoxyphenylhydrazone; PK, proteinase K; \( \phi \)-phe, orthophenanthrolin; DSP, dithiobis-succinimidylpropionate; AAC, ATP/ADP carrier; ANT, adenine nucleotide translocator.
Small Tim Complexes of Human Mitochondria

For import of integral inner membrane proteins the TIM22 complex interacts with a group of structurally related proteins located in the intermembrane space, the so named small Tim proteins (22–25). The small Tim proteins belong to an evolutionary conserved family of putative zinc finger proteins. They are thought to function as molecular chaperones that guide hydrophobic membrane proteins across the aqueous environment of the intermembrane space and maintain them in an import-competent conformation. In yeast, the small Tim proteins named Tim9, Tim10, and Tim12 specifically mediate the import and insertion of metabolite carriers into the inner membrane (25–27). They are organized in two distinct hetero-oligomeric complexes. A soluble 70-kDa complex presumably consisting of three molecules Tim9 and three molecules Tim10 associates with the incoming precursor still associated with the TOM-complex by binding to hydrophobic segments (28, 29); in a second complex one molecule Tim10 is substituted by one Tim12 (22). This complex is part of a 300-kDa high molecular mass complex which in addition comprises the membrane integral components of the TIM22 translocase. The membrane association of this Tim9–10–12 complex is mediated by Tim12 shown to directly interact with Tim22 (23). Upon releasing from the TOM complex the precursor proteins are passed on from the Tim9–10 to this membrane-associated Tim9–10–12 complex for insertion into the inner membrane (26). The inserted carriers assemble into functional dimers (30). The insertion of the hydrophobic preproteins into the inner membrane depends on the presence of a membrane potential but does not require ATP.

The family of small Tim proteins comprise two further proteins, Tim8 and Tim13 (31). In contrast to Tim9, Tim10 and Tim12 they are not essential for the viability of yeast. Tim8 and Tim13 are also organized in the intermembrane space in a hetero-oligomeric 70-kDa complex. In particular, they do not assist the import of the hydrophobic carrier proteins into the inner membrane. Recently, it was shown that both proteins effect import in vitro of non-carrier proteins of the inner membrane such as Tim23 (32–34). The Tim8–13 complex traps the incoming precursors in the intermembrane space thereby maintaining them in a translocation competent conformation.

In mammalian cells six members of the family of small Tims are expressed. All homologues of each Tim9 and Tim13 (human Tim9 and Tim13) and two homologues of each Tim10 (Tim10a and Tim10b) and Tim8 (DDP1 and DDP2) were described (21, 35). DDP1 is the deafness-dystonia peptide, which is mutated in Mohr-Tranebjaerg syndrome, a progressive neurodegenerative disorder characterized by sensorineural hearing loss, dystonia, mental retardation and blindness (31, 36). Homologues of Tim12 appear to be neither expressed in humans nor in lower eukaryotes except Saccharomyces cerevisiae.

All six homologues are ubiquitously expressed at the mRNA level in human organs excluding that the a and b variants of Tim8 and Tim10 represent tissue specific or developmental isoforms (21, 35). However, only five of these can be found at the protein level in human mitochondria (37). So far, all attempts failed to detect endogenous DDP2 although it can be excluded that it is a transcribed pseudogene (21). Yet, it is not clear whether DDP2 is an authentic protein that is expressed at levels that cannot be detected by conventional biochemical techniques or is expressed in specific types of cells. Recently, we have characterized the human components DDP1 and Tim13 in more detail. They form soluble 70-kDa hetero-oligomeric complexes in the mitochondrial intermembrane space and act in a manner similar to the yeast Tim8–Tim13 complex during import of Tim23 into the inner mitochondrial membrane (37–39). The differences found between humans and yeast in the requirement for functional DDP1–Tim13 versus Tim8–13 complexes during the import of precursors has been suggested as a molecular basis for the development of the Mohr–Tranebjaerg syndrome (37).

Here, we have investigated the structure and function of Tim9, Tim10a, and Tim10b, the human homologues of the essential small Tim proteins of yeast. We show that they also form two distinct hetero-oligomeric complexes, which are in contrast to the human DDP1–Tim13 tightly associated with the outer face of the inner mitochondrial membrane. Both complexes interact in a metal-dependent manner with the adenine nucleotide translocator during import and with Tim22 for insertion into the inner membrane.

EXPERIMENTAL PROCEDURES

Preparation of Mitochondria and Submitochondrial Fractions—Mitochondria from yeast cells were isolated as described before (40). Mitochondria from frozen Hela cells or mouse liver were prepared by homogenization in 0.25 M sorbitol, 0.1 mg/ml bovine serum albumin, 80 mM KCl, 10 mM Mvalinomycin and 500 μM CCCP. Mitochondria were resuspended in phosphate-buffered saline (PBS) buffer pH 7.2. For generation of a mitochondrial potential (Mitochondria were preincubated with 2.5 mM ATP, 5 mM NADH, and 2.5 mM succinate for 10 min at 25 °C. The membrane potential was dissipated by the addition of 1 mM valinomycin and 50 μM CCCP. The reactions contained 1 μg/μl of mitochondrial protein and 4% reticulocyte lysate containing the radio-labeled precursor protein. Aliquots corresponding to 50 μg of protein were immunoprecipitated using affinity-purified antibodies against human Tim9, Tim10a, Tim10b, or Tim22 prebound to protein A-Sepharose beads. The complexes were dissociated in SDS-containing sample buffer and analyzed by SDS-PAGE and Western blotting.

In Vitro Synthesis of Precursor Proteins and Import into Mitochondria—Precursor proteins were synthesized by coupled transcription/translation in rabbit reticulocyte lysate (Promega) in the presence of [35S]methionine using the respective cDNAs encoded on a pGEM4Z plasmid. Import reactions into mitochondria freshly prepared from mouse liver were carried out for 15–20 min at 25 °C in import buffer (0.6 M sorbitol, 0.1 mg/ml bovine serum albumin, 80 mM KCl, 10 mM Mg(OAc)₂, 2.5 mM EDTA, 2 mM KH₂PO₄, 50 mM HEPES-KOH, pH 7.2). For generation of a mitochondrial potential (Mitochondria were preincubated with 2.5 mM ATP, 5 mM NADH, and 2.5 mM succinate for 10 min at 25 °C. The membrane potential was dissipated by the addition of 1 μM valinomycin and 50 μM CCCP. The reactions contained 1 μg/μl of mitochondrial protein and 4% reticulocyte lysate containing the radio-labeled precursor protein. Aliquots corresponding to 50 μg of mitochondrial protein were incubated with protease (50–200 μg/ml proteinase K) for 20 min on ice followed by incubation with protease inhibitor (5 min on ice) or left untreated. For generation of mitoplasts the import assay was diluted in 10 volumes of swelling buffer (20 mM HEPES-KOH pH 7.4; 0.1% digitonin). Mitochondria were washed twice.
Small Tim Complexes of Human Mitochondria

RESULTS

The Human Mitochondrial Intermembrane Space Proteins Tim9, Tim10a, and Tim10b Are Tightly Associated with the Inner Membrane—Recently, we identified Tim9, Tim10a, and Tim10b as members of the family of small Tim proteins expressed in human mitochondria (21, 37). In order to analyze the sub mitochondrial location of these proteins in more detail human HeLa cells were treated in a stepwise manner with increasing concentrations of digitonin and fractionated by centrifugation (37). The outer membrane was selectively opened at concentrations of 0.08% digitonin with more than about 50% of the soluble intermembrane space protein DDP1 in the supernatant (Fig. 1A). DDP1 was entirely released at a concentration of 0.13% digitonin; the integral inner membrane Tim23 remained in the pellet fraction (Fig. 1A).

Under these conditions human Tim9, Tim10a, and Tim10b were still entirely found in the pellet fraction demonstrating that they are, in contrast to DDP1, associated with the inner membrane rather than mobile in the intermembrane space.

For cross-linking experiments, import reactions were performed in import buffer using 7.5% reticulocyte lysate with the radiolabeled precursor protein. A 50-μg aliquot of freshly isolated mouse liver mitochondria was used for total cross-links and 200 μg for cross-linking and subsequent immunoprecipitation. After import, dithiobis-succinimidyl-propionate (DSP) was added to a final concentration of 1 mM and subsequent immunoprecipitation. After import, dithiobis-succinimidyl-propionate was added to a final concentration of 1 μM and incubated 30 min on ice. The cross-linking reaction was quenched with 100 mM glycine pH 8.0. Mitochondria were either analyzed directly by SDS-PAGE, Western blotting, and immunodecoration with antibodies against Tim9, Tim10a, and Tim10b or solubilized in lysis buffer (15 mM Tris pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 0.05% SDS) for 30 min on ice and subjected to immunoprecipitation. Where indicated preincubation with 10 μg EDTA and 2 mM orthophenanthroline (o-phen) was carried out prior to import reactions for 1 h at 4 °C.

Antibodies—Antisera against the C termini of human small Tim proteins (Tim9, Tim10a, Tim10b, DDP1) were raised in rabbits as described before (37). Antisera against human Tim23 were raised in rabbits using a chemically synthesized peptide (CFDPKDPYRTPTAKE). Antibodies were affinity purified on SulfoLink gel (Pierce). Monoclonal antibody against Tim23 was purchased from BD Biosciences.

The Human Mitochondrial Intermembrane Space Proteins Tim9, Tim10a, and Tim10b Are Associated with the Mitochondrial Inner Membrane. A, controlled opening of the mitochondrial outer membrane. Human mitochondria from HeLa cells (50 μg) were incubated with increasing concentrations of digitonin (Dig). The membranes were separated from the soluble content by centrifugation. Membrane pellets (P) and trichloroacetic acid-precipitated supernatants (S) were analyzed by SDS-PAGE and Western blotting using antibodies against Tim9, Tim10a, and Tim10b. Anti-DDP1 and anti-Tim23 were used as controls. B, membrane association of Tim9, Tim10a, and Tim10b. Mitoplasts were generated by incubation of mitochondria (50 μg) in 0.1% digitonin and resuspended in phosphate buffer, phosphate buffer containing 500 mM NaCl or phosphate buffer containing 100 mM Na₂CO₃ (pH 11). Membranes (P) and soluble fractions (S) were separated by centrifugation at 100,000 × g and analyzed by SDS-PAGE, Western blotting, and immunodecoration with antibodies against Tim9, Tim10a, and Tim10b.

The distribution between supernatant and pellet fraction was not influenced by whether a membrane potential across the inner membrane was present or not (data not shown). At a concentration of 0.3% digitonin Tim9, Tim10a, Tim10b, as well as the membrane protein Tim23 were released into the supernatant.

To assess the strength of the membrane association, the outer membrane of mitochondria was selectively opened using 0.1% digitonin. Subsequently, the mitochondria were fractionated in the presence of high salt (500 mM NaCl) or carbonate extraction buffer (pH 11.5). About one-half of Tim9 and Tim10a was recovered in the supernatant upon high salt treatment; both proteins were completely released from the membrane upon carbonate extraction (Fig. 1B). A significant portion of Tim10b could only be released upon treatment with alkaline pH but not with high salt buffer. As expected, the integral inner membrane protein Tim23 was not extracted from the membrane by either condition (Fig. 1B). These results demonstrate that the human intermembrane space proteins Tim9, Tim10a, and Tim10b are, in contrast to their yeast counterparts, more tightly associated with the outer face of the inner membrane. Tim10b exhibits the highest affinity to the membrane and resembles in its behavior that of yeast Tim12.

The Small Tim Proteins Are Organized in Two Distinct Complexes in Human Mitochondria—To assess the organization of Tim9, Tim10a, and Tim10b, mitochondria were lysed with 0.5% digitonin and co-immunoprecipitations were performed using affinity purified antibodies. Immunoblot analysis of precipitated material showed that antibodies against Tim9 precipitated both Tim9 and Tim10a; likewise, both proteins were precipitated using antibodies against Tim10a indicating that both proteins interact in a complex (Fig. 2). Both antibodies depleted the cognate antigen as well as their partner protein from mitochondrial extracts. Anti-Tim9 and anti-Tim10a also quantitatively precipitated Tim10b from mitochondrial extracts; whereas, anti-Tim10b only partially precipitated Tim9 and Tim10a. A fraction of Tim9 and Tim10a remained in the supernatant after precipitation (Fig. 2). Together, these results suggest the presence of two distinct hetero-oligomeric complexes in the intermembrane space. One complex consists of Tim9 and Tim10a. A second complex additionally contains Tim10b. As a rough estimation this complex accounts for about 20–30%.

To assess the organization of these two complexes a gel filtration analysis was performed on a Superose-12 column. The HeLa cell mitochondria were solubilized with digitonin that preserves the TIM22 complex in yeast (16, 23). Tim10b
exclusively eluted from the column in a fraction corresponding to an apparent molecular mass of ~450 kDa (Fig. 3, A and B, left panels). In contrast, only about 30% of Tim9 and Tim10a eluted in the 450 kDa peak fractions; the remaining portion of these proteins eluted corresponding to a molecular mass ~70 kDa. When solubilized with 0.5% Triton X-100, the yeast TIM22 complex destabilizes and Tim12 dissociates from Tim22. Under these conditions also the human proteins were exclusively found in the fractions corresponding to the 70-kDa species (Fig. 3, A and B, right panels).

This suggests that there are two forms of 70-kDa complexes that are composed of different subunits. The Tim9-10a-10b complex is tightly associated with the mitochondrial inner membrane and is part of a high molecular weight complex of 450 kDa. The majority of Tim9 and Tim10a is found in a distinct complex, Tim9-10a complex, that lacks Tim10b. The strength of the association of the Tim9-10a complex with the inner membrane appears to be weaker than that of the Tim9-10a-10b complex.

Tim9, Tim10a, and Tim10b Interact with Human Tim22—In yeast, the association of the TIM9-10-12 complex with the inner membrane is mediated by an interaction between Tim12 and the membrane-integral component Tim22. Recently, we identified the human Tim22 homologue (21). Like its yeast counterpart, human Tim22 is embedded in the mitochondrial inner membrane of human mitochondria. We asked whether the human small Tim proteins are in direct contact with Tim22. Human mitochondria were therefore solubilized with 0.5% digi-
miton and immunoprecipitations were performed with antibodies against Tim9, Tim10b, and Tim22. Western blot analysis of the immunoprecipitated material showed that both anti-Tim9 and anti-Tim10b were able to co-precipitate Tim22 (Fig. 4). However, only a minor fraction of total Tim22 was precipitated with these antibodies whereas anti-Tim22 was able to deplete Tim22 from mitochondrial extracts. Likewise, only about 20% of each Tim9, Tim10a, and Tim10b was precipitated with antibodies against Tim22 (Fig. 4). These data indicate that the human small Tim proteins are in contact with human Tim22. However, only a small fraction of the Tim9-10a-10b complex, and maybe also of the Tim9-10a complex, appears to interact with human Tim22 in a stable manner. Thus, in human mitochondria there is no stable complex containing Tim9-10a-10b together with Tim22. These data are supported by the observation that human Tim22 is not found in the 450-kDa peak elution fraction after gel filtration (data not shown) that contains all of Tim10b and parts of Tim9 and Tim10a.

**The Human Small Tims Act During Import of Carrier Proteins into the Inner Membrane**—We further investigated the functional role of the small Tim proteins in human mitochondria and asked whether they act during import of metabolite carriers into the inner membrane of mammalian mitochondria. Mitochondria were freshly prepared from mouse liver and energized with NADH and succinate or preincubated with valinomycin/CCCP to dissipate the membrane potential \( \Delta \psi \). Radiolabeled precursor of the human ANT3, the liver-specific isoform of the mammalian adenine nucleotide translocators, was added and import into mitochondria was quantified. In the presence of a membrane potential \( \Delta \psi \), a significant portion of human ANT3 was imported into mitochondria indicated by the band resistant to protease treatment with proteinase K (PK) (Fig. 5A). In the absence of \( \Delta \psi \), the amount of imported ANT3 was severely reduced. The import of ANT3 into mitochondria isolated from yeast cells was inefficient (data not shown).

To assess the insertion of the human carrier into the inner membrane, radiolabeled ANT3 was imported into mammalian mitochondria and treated with PK after selective opening (see above) of the outer membrane. Under these conditions, most of imported ANT3 remained resistant against PK (Fig. 5B, left panel). The efficiency of membrane insertion of ANT3 into mammalian mitochondria was comparable to that of yeast AAC2 into mitochondria isolated from yeast (Fig. 5B, right panel). In contrast to yeast AAC2, ANT3 could not be clipped to a lower size fragment even at high concentrations of PK (200 \( \mu \)g/ml) (Fig. 5B). This might be due to the fact that ANT3 contains very short hydrophilic stretches at both termini and that these lack putative cleavage sites.

**Fig. 4. Human small Tim proteins interact with Tim22.** HeLa cell mitochondria corresponding to 50 \( \mu \)g of protein were solubilized in 0.5% digitonin and subjected to immunoprecipitation with anti-Tim9, anti-Tim10b, and anti-Tim22. Immunoprecipitates were analyzed by SDS-PAGE and Western blotting using antibodies against Tim9, Tim10a, and Tim22.

**Fig. 5. Import of human ANT3 into mouse liver mitochondria.** A, import of ANT3 is dependent on a membrane potential \( \Delta \psi \). Radiolabeled ANT3 was synthesized in rabbit reticulocyte lysate and incubated for 15 min with mouse mitochondria in the presence or absence of a membrane potential \( \Delta \psi \). Mitochondria were treated with PK (200 \( \mu \)g/ml) or left untreated. Samples were analyzed by SDS-PAGE. B, membrane-inserted AAC2, but not ANT3 is clipped to a lower size fragment by PK (indicated by an arrowhead). Radiolabeled ANT3 or AAC2 was incubated with mitochondria for 15 min in the presence of a membrane potential. Mitoplasts (MP) were generated by incubation of mitochondria in 0.1% digitonin and digested with PK (200 \( \mu \)g/ml) or left untreated. C, ANT3 precursor specifically cross-links to Tim9 and Tim10a. De-energized mitochondria were incubated with radiolabeled ANT3 for 20 min. Mitochondria were re-isolated and 1 mM DSP was added where indicated. Mitochondria were solubilized with 0.5% Triton X-100 in the presence of 0.05% SDS and subjected to immunoprecipitation with anti-Tim9 or anti-Tim10a. Nonimmune serum was used as a control. The ANT3-specific cross-linking product is indicated by an arrowhead. D, isolated mitochondria were pretreated with CCCP/valinomycin to dissipate the membrane potential or membrane potential was generated with succinate and NADH. Mitochondria were pre-treated with EDTA/o-phen where indicated and incubated with radiolabeled ANT3 for 20 min. Cross-linking was performed with 1 mM DSP. Samples were lysed with 0.5% Triton X-100 and 0.05% SDS and subjected to immunoprecipitation with Tim9 antibody. The ANT3-specific cross-link is indicated by an arrowhead. Samples were analyzed by SDS-PAGE, Western blotting and autoradiography.

To address whether the human small Tims directly interact with translocation intermediates of carriers during import into mammalian mitochondria, human ANT3 was subjected to
Small Tim Complexes of Human Mitochondria

Chemical cross-linking. To accumulate translocation intermediates in the intermembrane space (stage III), mouse liver mitochondria were preincubated with valinomycin and CCCP to dissipate the membrane potential. Radiolabeled ANT3 precursor was added and cross-linking with DSP was performed. Mitochondria were lysed and subjected to immunoprecipitation with antibodies against Tim9 and Tim10a. With both antibodies one specific cross-linking adduct was detected corresponding to an apparent molecular mass of about 40 kDa (Fig. 5C). This cross-link adduct was not observed when nonimmune serum was used for immunoprecipitation or when import was performed in the presence of a membrane potential $\Delta \psi$. These data indicate that Tim9 and Tim10a are in direct contact with ANT3 precursor during import. Additionally, cross-link products of higher molecular sizes were observed when anti-Tim10a was used for precipitation. These may reflect the oligomeric organization of the small Tim proteins.

The small Tim proteins of eukaryotes contain four conserved cysteine residues that constitute a metal binding site and are required for the formation of typical zinc finger structures. In yeast, the interaction of Tim10 and Tim12 with AAC2 precursors during import is dependent on the presence of divalent metal ions (23). Here, we tested whether metal ions are also required during import of human carrier proteins. Mitochondria isolated from mouse liver were de-energized with valinomycin/CCCP and incubated with radiolabeled ANT3 in the presence or absence of the metal chelating agents EDTA and o-phenanthroline (o-phen). Subsequently, cross-linking with DSP was performed. Immunoprecipitation with anti-Tim9 revealed a specific cross-link adduct of about 40 kDa in the presence of divalent cations but not in their absence (Fig. 5D). Furthermore, the cross-link adduct was not observed when the import was performed in the presence of a membrane potential. Thus, metal ions are required for the interaction of the human Tim9-10a and Tim9-10a-10b complexes with ANT3 during its import into mitochondria. This dependence reflects the requirement of zinc ions for the formation of zinc finger domains of small Tim proteins.

Discussion

Here, we investigated the structural features and functional role of the human intermembrane space components Tim9, Tim10a, and Tim10b. They belong to the evolutionary conserved family of small Tim proteins, which in mammals additionally harbor DDP1 and Tim13 (21, 37). All these proteins share a characteristic Cys$_2$ metal binding motif that is required for the formation of zinc finger-like structures.

Based on amino acid sequence comparison, human Tim9 and Tim10a were shown to represent the homologues of yeast Tim9 and Tim10; the degrees of similarity between human and yeast components were 60 and 62%, respectively. Only the human Tim10b shows a significantly lower degree of overall similarity (–40%) to yeast Tim9, Tim10, and Tim12. Amino acid sequence analysis could not assign human Tim10b to any of these components; it was classified as a second Tim10 homolog only on the basis of a phylogenetic analysis focusing on the Cys$_2$ domain (21). Thus, a clear-cut Tim12 homolog seems to be absent in mammals and other eukaryotes except S. cerevisiae. This is surprising because the yeast Tim12 fulfills a specific and essential role during the import of mitochondrial carriers that can be assumed to be conserved in higher eukaryotes (23, 25, 26). Consequently, we asked which of the human small Tim proteins fulfill a function similar to that of yeast Tim12.

We showed that the human small Tims investigated here are organized in two hetero-oligomeric complexes in the mitochondrial intermembrane space. One complex contains Tim9 and Tim10a; its apparent molecular size is about 70 kDa. The second contains Tim9, Tim10a, and Tim10b and is part of a higher molecular mass complex of about 450 kDa. About two-thirds of Tim9 and Tim10a are found in the Tim9-10a complex; while the remaining Tim9 and Tim10a is associated with Tim10b in the 450 kDa complex. In yeast, also two hetero-oligomeric complexes of different sizes are found in the intermembrane space: a TIM9-10 and a TIM9-10-12 complex. Similar to mammalian mitochondria, one complex, TIM9-10, exists in a 70-kDa form, whereas the second complex, TIM9-10-12, is part of a larger complex of 300 kDa (22–25). Thus, the distribution of the human small Tims among the two complexes resembles that of yeast. Moreover, the data suggest that the Tim10b might be the functional homolog of yeast Tim12 since both, yeast Tim12 and human Tim10b are exclusively found in the large complexes and not in the 70-kDa complexes.

Accordingly, we asked whether the human Tim10b mediates the association of the human small Tim complexes with the inner membrane in a manner similar to yeast Tim12. Indeed, Tim10b exhibits a higher membrane affinity when compared with Tim9 and Tim10a. It remained membrane associated even at high ionic strength whereas Tim9 and Tim10a were released under the same conditions. Surprisingly, Tim9 and Tim10a appear to be quantitatively associated with the outer face of the inner membrane. Thus, in contrast to yeast, the intermembrane space of mammalian mitochondria appears to lack soluble 70-kDa complexes composed of Tim9 and Tim10a.

This observation was unexpected since the soluble TIM9-10 complex of yeast is thought to function as a trans-site receptor for incoming carriers still associated with the TOM complex (2, 26, 29). It escorts the hydrophobic precursors across the intermembrane space to the TIM22 translocase in the inner membrane. The function of these soluble 70-kDa complexes appears not to be essentially required for import of carriers into yeast mitochondria. In a mutant yeast strain (Δ70k) that lacks soluble TIM9-10 complexes in the intermembrane space, import of AAC into mitochondria was found 2–3-fold reduced (42). However, the steady-state levels of AAC in Δ70k mitochondria remained unaffected. This Δ70k strain contains minimal levels of Tim9 and Tim10 that confer viability to the yeast cells; both proteins were found in tight association with the inner membrane. Thus, in yeast the soluble 70-kDa complexes facilitate the import of AAC although their presence is not essentially required (42). It appears that their function is not conserved between lower and higher eukaryotes and import of carrier proteins into mammalian mitochondria is efficient even in the absence of the soluble 70-kDa complexes. Therefore, other mechanisms that prevent the hydrophobic carriers from aggregation in the aqueous intermembrane space must have evolved in mitochondria of higher eukaryotes.

In yeast, the precursors are transferred to the putative insertion pore of the TIM22 translocase via the tightly bound TIM9-10-12 complex. We have shown that at least a small fraction of the Tim9-10a-10b, and maybe the Tim9-10a complex, is in contact with Tim22 in human mitochondria. This indicates that the human intermembrane space complexes are indeed involved in the TIM22 import pathway. These data also imply that the major portion of the Tim9-10a-10b complex is not stable associated with human Tim22. Notably, the human 450 kDa Tim9-10a-10b complex does not contain Tim22. This finding is in contrast to yeast, where all of TIM9-10-12 is found in a stable complex of 300 kDa together with Tim22, Tim54, and Tim18 (23, 25). Based on these observations it can be speculated that human Tim9-10a-10b interacts with the membrane-integral Tim22 in a dynamic manner when the carrier precursors are transferred to the putative pore of TIM22 complex for proper insertion into the inner membrane. Further-
more, human homologues of yeast Tim54 and Tim18 appear not to be expressed in higher eukaryotes indicating that also the structural organization of the membrane-integral part of the human TIM22 complex differs remarkably from that of the fungal complex.

We provided evidence that the human intermembrane space complexes are in contact with carrier precursors during import into mammalian mitochondria suggesting that they act as specific chaperones for metabolite carriers in humans. However, our data suggest that different mechanisms and specific requirements for import and insertion of carrier preproteins into the inner membrane have evolved in higher eukaryotes. This conclusion is additionally supported by the observation that the human ANT3 cannot be imported into yeast mitochondria whereas the yeast AAC is efficiently imported into mitochondria of either yeast or mammals. Furthermore, the tight membrane association of the small Tim complexes implies the development of a different mechanism that ensures the maintenance of import competence of hydrophobic carriers during their import. It remains to be clarified which further components contribute to the TIM22 import pathway and allow to fulfill the specific import requirements of the mammalian carrier proteins.

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