Role of the Deafness Dystonia Peptide 1 (DDP1) in Import of Human Tim23 into the Inner Membrane of Mitochondria*

Received for publication, June 8, 2001, and in revised form, July 16, 2001
Published, JBC Papers in Press, August 6, 2001, DOI 10.1074/jbc.M105313200

Uli Rothbauer‡§, Sabine Hofmann‡§, Nicole Mühlenbein‡, Stefan A. Paschen¶, Klaus-Dieter Gerbitz‡, Walter Neupert¶, Michael Brunner¶, and Matthias F. Bauer‡§

From the ‡Institut für Klinische Chemie, Molekulare Diagnostik und Mitochondriale Genetik, Akademisches Lehrkrankenhaus München-Schwabing, Koelner Platz 1, München 80804 and the ¶Institut für Physiologische Chemie der Universität München, Butenandstrasse 5, München 81377, Germany

Tim8 and Tim13 of yeast belong to a family of evolutionary conserved zinc finger proteins that are organized in hetero-oligomeric complexes in the mitochondrial intermembrane space. Mutations in DDP1 (deafness dystonia peptide 1), the human homolog of Tim8, are associated with the Mohr-Tranebjaerg syndrome, a progressive neurodegenerative disorder. We show that DDP1 acts with human Tim13 in a complex in the intermembrane space. The DDP1-hTim13 complex is in direct contact with translocation intermediates of human Tim23 in mammalian mitochondria. The human DDP1-hTim13 complex complements the function of the Tim8-13 complex in yeast and facilitates import of yeast and human Tim23. Thus, the pathomechanism underlying the Mohr-Tranebjaerg syndrome may involve an impaired biogenesis of the human Tim23 complex causing severe pleiotropic mitochondrial dysfunction.

The vast majority of mitochondrial proteins are encoded as precursors in the nuclear genome. Mitochondrial biogenesis is, therefore, dependent on the import and sorting of the nuclear encoded precursor proteins into mitochondrial subcompartments. In eukaryotes three distinct preprotein import systems located in the mitochondrial outer and inner membrane have been described (1–5). The outer membrane contains a general preprotein translocase, the TOM complex, which mediates the recognition and binding of preproteins and their transfer across the outer membrane. This complex is most likely used by all nuclear encoded precursors. Import into and across the inner membrane is mediated by two distant inner membrane translocases, the TIM22 and the TIM23 complexes. Both TIM complexes cooperate with the TOM complex but differ in their substrate specificity (6–11). The TIM23 complex mediates import of preproteins with a positively charged matrix targeting signal into the mitochondrial matrix space and into the inner membrane (6, 12, 13). The translocation of such precursors into the matrix requires the membrane potential Δψ across the inner membrane and ATP in the matrix. The Δψ drives the translocation of the presequences through the protein-conducting channel of the TIM23 complex which is formed by the membrane-integrated proteins Tim23 and Tim17 (6, 12). A molecular motor that is attached to the inner side of this channel then promotes further translocation of the mature portion of the preproteins into the matrix. This motor consists of the peripheral membrane protein Tim44, the mitochondrial Hsp70, and the nucleotide exchange factor Mge1. Together, these components in repeated ATP-dependent reaction cycles facilitate the vectorial translocation into the matrix in a stepwise manner (14).

The TIM22 complex mediates the insertion of a class of hydrophobic proteins with internal targeting signals into the inner membrane (7–11, 15–17). Typical substrates are members of the mitochondrial carrier family and other integral inner membrane proteins that are synthesized without a matrix-targeting signal. Insertion of these precursors into the inner membrane is strictly dependent on Δψ but does not require ATP in the matrix.

The transfer of carrier proteins from the TOM complex to the TIM22 complex involves the assistance of three small, structurally related proteins of the intermembrane space, Tim9, Tim10, and Tim12 (8, 10, 11, 15, 17). These proteins belong to an evolutionary conserved family of zinc finger proteins characterized by a Cys4 motif (5, 18). Tim9 and Tim10 form a hetero-oligomeric complex of 70 kDa which interacts with translocation intermediates of the precursors which are partially translocated across the TOM complex (10, 15). From the Tim9-10 complex the carrier proteins are handed over to the Tim9-10-12 complex tightly associated with the TIM22 complex in the inner membrane. The insertion of the translocation intermediate into the inner membrane by the TIM22 complex is strictly dependent on the membrane potential Δψ.

The family of small zinc finger proteins comprises two further components, Tim8 and Tim13 (18–20). Their precise function was not known until recently. In the yeast Saccharomyces cerevisiae, Tim8 and Tim13 are also organized in a hetero-oligomeric complex in the intermembrane space. They are, in contrast to Tim9, Tim10, and Tim12, not essential for the viability of yeast cells (19). Recently it was shown that the Tim8-13 complex of yeast interacts with translocation intermediates of Tim23, the major component of the translocase for matrix-targeted preproteins (17, 21, 22). The Tim8-13 complex assists the import of Tim23 by binding to the partially trans-
located precursor in the intermembrane space still associated with the TOM complex. It is only strictly required when the membrane potential is low and the interaction with TIM22 complex is inefficient (21). Under these conditions the TIM8-13 complex was proposed to function in accumulating the Tim23 translocation intermediate thereby shifting the equilibrium toward direct interaction with the membrane-integrated portion of the TIM22 complex (21).

The human homolog of Tim8 is encoded by the DDPI (deafness dystonia peptide 1) gene. Mutations in the DDPI cause the Mohr-Tranebjærg syndrome, a progressive neurodegenerative disorder characterized by sensorineural hearing loss, dystonia, mental retardation, and blindness (19, 23, 24). Most of the DDPI mutations are loss-of-function mutations predicted to lead to an absent or a truncated gene product. So far, only one missense mutation was found causing a cysteine to tryptophan exchange (C66W) within the Cys4 motif (25).

In the present study, we analyzed the structural organization of the human zinc finger proteins DDPI and hTim13 and their functional role in mitochondrial preprotein import. We show that DDPI interacts with hTim13 in the mitochondrial intermembrane space thereby forming a hetero-oligomeric complex of 70 kDa. When expressed in yeast, the DDPI-hTim13 complex facilitates import of hTim23. In human mitochondria the DDPI-hTim13 complex is in direct contact with translocation intermediates of hTim23. This suggests that an impaired mitochondrial preprotein import is the pathogenic basis of the Mohr-Tranebjærg syndrome.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Strain Construction**—For subcloning DDPI and hTim13 into expression vectors, DNA was polymerase chain reaction amplified from DDPI- and hTim13-specific expressed sequence tag clones (18) using specific oligonucleotide primers (DDPI forward: 5′-atg gat tcc tcc tct ccc-3′; DDPI reverse: 5′-tca ggt gta gaa ggt ctc g-3′; hTim13 forward: atg gag ggc gtc ccc-3′; hTim13 reverse: 5′-tca gtt gcc tgc-3′). The DDPI and hTim13 polymerase chain reaction products were digested with EcoRI and HindIII and subcloned into pYX142/LEU2 and pYX232/TRP1 yeast expression vectors (Novagen), respectively. The S. cerevisiae strain MB2-32/13 (tim8::HIS3, tim13::URA3) (21) was transformed with both plasmids, and transformants were selected on minimal medium without histidine, uracil, leucine, and tryptophan. MBP-fusion proteins were generated by digestion of the DDPI and hTim13 polymerase chain reaction products with BamHI and XbaI and subcloning into pMal-cRI vectors (New England Biolabs). For *in vitro* synthesis of precursor proteins, yeast Tim23 and human TIM13 DNA were digested with EcoRI/HindIII and subcloned into pGEM4 (21).

**Preparation of Mitochondria and Subcellular Fractions**—Mitochondria from yeast were isolated as described (26). Mitochondria from frozen HeLa cells (Computer Cell Culture Center, Mons, Belgium) or mouse liver were prepared in a medium containing 0.25M sucrose, 5 mM TRIS/HCl, pH 7.4, by differential centrifugation and subsequent sucrose step gradient ultracentrifugation as described previously (27). To analyze the subcellular localization of human small Tim proteins, the postmitochondrial supernatant after a 10,000 × *g* centrifugation step was centrifuged to a TL100 ultracentrifuge at 100,000 × *g* and 4 °C for 30 min to yield the cytoplasmic fraction (supernatant) and the microsomal fraction (pellet). To release soluble components of the intermembrane space into the supernatant, mitochondria prepared from HeLa cells were solubilized using increasing concentrations of digitonin (0.05–0.3% w/v or 0.75–4.5 μg of digitonin/μg of mitochondrial protein) and centrifuged at 100,000 × *g* for 30 min.

**Size Determination of the Native Complex**—Human mitochondria (100–250 μg) were solubilized with 0.5% Triton X-100 in 0.5 ml of column buffer (1 × phosphate-buffered saline, pH 7.4, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride) for 30 min at 0 °C. After centrifugation at 100,000 × *g* in a Beckman TL100 ultracentrifuge (30 min), the clear supernatant was either analyzed by blue native gel electrophoresis (100 μg/lane) (28, 29) and subsequent immunodetection or subjected to a Superose-12 gel filtration column (250 μl) (Amersham Pharmacia Biotech) and chromatographed at a flow rate of 0.15 ml/min in column buffer containing 0.5% Triton X-100. Fractions (0.5 ml each) were analyzed by SDS-PAGE and immunoblotting with antibodies against DDPI and hTim13. As calibration standards thyroglobulin (670 kDa), apoferritin (440 kDa), β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa) were used.

Fractions in which DDPI and hTim13 coeluted were subsequently subjected to immunoprecipitation using either affinity-purified anti-DDPI or anti-hTim13 IgG. Prior to immunoprecipitation, Triton X-100 was diluted to a final concentration of 0.2%. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting using antibodies against DDPI and hTim13.

**Immunoprecipitation**—Isolated mitochondria were resuspended at a concentration of 0.2 mg/ml in 1 ml of column buffer (1 × phosphate-buffered saline, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride) with 0.5% Triton X-100 and lysed on ice for 30 min. After a clarifying spin (30 min, 100,000 × g), the extracts were split into two aliquots, diluted to a final concentration of Triton X-100 of 0.2%, and immunoprecipitated using antibodies against either DDPI or hTim13 prebound to protein A-Sepharose beads. The immunocomplexes were dissociated in SDS-containing sample buffer and analyzed by SDS-PAGE and immunoblotting with anti-DDPI and anti-hTim13 antibodies.

**Determination of Zinc**—MBP-DDPI, MBP-hTim13, and MBP-lacZ fusion proteins were expressed in XL1-blue *E. coli* cells at 30 °C in the presence of 1 mM zinc acetate for 16 h. Cells were lysed by sonication in a buffer containing 1 mM zinc acetate, 20 mM HEPES/KOH (pH 7.2), 100 mM NaCl, 20 mM β-mercaptoethanol. A clarifying spin was performed, and the supernatant was either incubated with 40 mM N-ethylmaleimide (NEM) for 1 h or applied directly to an amyllose column for affinity purification. After binding the column was washed with 50 ml of lysis buffer lacking the zinc acetate. Elution from the resin was obtained using 10 mM maltose. Concentrations of the eluted proteins were adjusted to 300 μg/ml, and the Zn²⁺ content was analyzed using inductively coupled plasma atomic emission spectroscopy (ICP) in a Varian-Vista Simultan spectrometer.

**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 (30). Import reactions into isolated yeast mitochondria or mitochondria prepared from mouse liver were carried out for 10–20 min at 25 °C in 180 μl of import buffer (0.6 mM 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) in the presence of 2.5 mM ATP and 5 mM NADH. To energize isolated mitochondria from mouse liver 2.5 mM succinate was added additionally. The reactions contained 180 μg of mitochondrial protein and 4% reticulocyte lysate with the radiolabeled precursor protein. Membrane potential was dissipated by omission of NADH and preincubation for 5 min at 25 °C with 1 mM valinomycin and 25 μM FCCP. Aliquots (50 μl) each corresponding to 50 μg of mitochondrial protein were either incubated with protease (50 μg/ml trypsin or 30 μg/ml proteinase K) for 20 min on ice followed by incubation with protease inhibitor (5% on ice) or left untreated. Mitochondria were washed twice in H5 buffer (20 mM HEPES/KOH, pH 7.4, 0.6 mM sorbitol), subjected to SDS-PAGE, and blotted onto nitrocellulose membranes.

For cross-link experiments, import reactions were performed in import buffer using 7.5% reticulocyte lysate with the radiolabeled precursor protein. A 100-μg aliquot of freshly isolated mouse liver mitochondria was used for total cross-links and 30 μg for cross-linking and subsequent immunoprecipitation. After import, n-maleimidobenzyloxy-N-hydroxy succinimide ester (MBS) was added to a final concentration of 100 μM and incubated 20 min on ice. The cross-linking reaction was quenched with 80 mM Tris, pH 8.0. Mitochondria were either analyzed directly by SDS-PAGE (total cross-links) or lysed in 0.5% Triton X-100 (30 min on ice) and subjected to immunoprecipitation.

**Antibodies**—Antisera against the C termini of human small Tim proteins were raised in rabbits by injecting the chemically synthesized peptides CKSKPVFSESLSD (DDPI), CYNQRSRERANM (hTim13), CAAAQGQVPSGSG (hTim10a), and CAAECPQWPSGPS (hTim10b) which had been coupled to activated keyhole limpet hemocyanin (Pierce). Antibodies were affinity purified on SulfoLink gel (Pierce). Polyclonal antisera against the C-terminal 13-mers of human Tim44 and human Tim23 were raised as described before (27).

**RESULTS**

DDPI and hTim13 are Soluble Components of the Mitochondrial Intermembrane Space—The identification of DDPI,
Human HeLa cell homogenates were fractionated into cytosol, microsomal fraction, and mitochondrial fraction. Equal amounts were subjected to SDS-PAGE and analyzed by immunoblotting, using affinity-purified antibodies against hTim9, hTim10a, hTim10b, hTim13, DDP1, Grp78, and antisera against hTim23 andargininosuccinate lyase (ASL). B, subcellular localization of the small Tim proteins. Isolated mitochondria from HeLa cells were treated with 0.1% digitonin (Fig. 1B), and hTim23, a component of the inner membrane (Fig. 1B), and hTim44, a peripheral mitochondrial matrix protein, were recovered in the pellet fraction (Fig. 1, B and C). Under these conditions DDP1 and hTim13 were completely released into the supernatant, demonstrating that both proteins are located in the intermembrane space.

**DDP1 and hTim13 Are Zinc-binding Proteins**—DDP1 and hTim13 belong to an evolutionary conserved protein family whose members share four conserved cysteine residues (Cys$_4$ motif) (5, 18). The Cys$_4$ motif is considered to constitute a metal binding site. The cysteine residues of yeast Tim10 and Tim12 have previously been shown to bind zinc, and zinc binding is required for the interaction of Tim10 and Tim12 with ADP/ATP carrier during import (8).

We constructed chimeric proteins consisting of DDP1 or of hTim13 fused to the MBP. The recombinant proteins were expressed in E. coli in the presence of 1 mM Zn$^{2+}$ ions and affinity purified on amylose resin (Fig. 2A). As a control, MBP protein fused to the α-fragment of the β-galactosidase (MBP-α) was purified (Fig. 2A). MBP-DDP1, MBP-hTim13, and MBP-α were analyzed using ICP. The atomic emission was determined at the zinc-specific wavelengths of 206.200 and 213.857 nm. The Zn$^{2+}$ content was calculated based on the counts obtained at the major wavelength at 213.857 (Fig. 2B). The preparations of MBP-DDP1 and MBP-hTim13 contained Zn$^{2+}$ in ~1:1 stoichiometry (Fig. 2C). In contrast, the control preparation of MBP-α contained only background levels of zinc. This demonstrates that DDP1 and hTim13 are zinc-binding proteins.

To test whether cysteine residues are involved in Zn$^{2+}$ binding the sulphydryl groups of MBP-DDP1 were modified with NEM prior to affinity purification. The Zn$^{2+}$ content of NEM-treated MBP-DDP1 was reduced to almost background levels (Fig. 2, B and C). Thus, the Cys$_4$ motif appears to mediate the binding of Zn$^{2+}$.

**DDP1 and hTim13 Are Organized in Hetero-oligomeric Complexes**—Using recombinant MBP-DDP1 and MBP-hTim13 for calibration, we estimated that mitochondria from HeLa cells contain approximately equal amounts of DDP1 and hTim13, corresponding to about 10–50 pg/μg of mitochondrial protein (Fig. 3A). To assess the organization of DDP1 and hTim13, mitochondria were lysed with 0.5% Triton X-100 and subjected to gel filtration analysis on a Superose-12 column. DDP1 and hTim13 coeluted in fractions corresponding to an apparent molecular mass of ~70 kDa (Fig. 3B). DDP1 and hTim13 were absent in fractions corresponding to higher molecular masses. In addition, mitochondria were analyzed by blue native gel electrophoresis (28, 29). DDP1 and hTim13 were monitored by immunoblotting with affinity-purified antibodies. Each antibody detected a major band with an electrophoretic mobility corresponding to a native molecular mass of ~70 kDa (Fig. 3C). A minor fraction of hTim13 was found in a complex of approximately 110 kDa. Thus, DDP1 and hTim13 appear to be organized in complexes of 70 kDa.

According to the predicted masses of DDP1 and hTim13 these complexes may be composed of six or seven subunits. To investigate whether DDP1 and hTim13 form homo- or heterooligomeric complexes, coimmunoprecipitations were performed using either total mitochondria or the 70-kDa fraction from the gel filtration column (Fig. 3D). When total mitochondria were lysed with Triton X-100, antibodies against DDP1 precipitated both DDP1 and hTim13. Likewise, antibodies against hTim13
Role of Human DDP1 and Tim13 in the Biogenesis of Tim23

precipitated hTim13 and DDP1 (Fig. 3D, left panel). Antibodies against DDP1 and hTim13 did not precipitate hTim9, hTim10a, or hTim10b (not shown). Both antibodies depleted their cognate antigen and the corresponding partner protein from mitochondrial extracts. Yet, Western blot analysis detected significantly lower amounts of the partner proteins in the immunoprecipitates. When DDP1 and hTim13 were immunoprecipitated directly from the 70-kDa fraction of the gel filtration column using relative amounts of the respective partner proteins were determined (Fig. 3D, right panel). This suggests that DDP1 and hTim13 are organized in heterohexameric complexes containing approximately equal amounts of each component. The lower efficiency of coprecipitation of the respective partner proteins is likely to result from dissociation of the complex during immunoprecipitation or the subsequent washing procedures.

DDP1 and hTim13 Are Orthologs of Yeast Tim8/Tim13—Tim8 and Tim13, the yeast homologs of DDP1 and hTim13, are not essential for cell viability. The fungal TIM8-13 complex interacts with the translocation intermediate of Tim23 and facilitates its import by trapping the incoming precursor in the intermembrane space thereby preventing retrograde translocation (21). Yeast cells carrying disruptions of TIM8 and TIM13 (Δ8/Δ13) grew normally at 30 °C and 15 °C in media containing the nonfermentable carbon source glycerol. In media containing glucose as a carbon source Δ8/Δ13 grew significantly slower at 15 °C (cold-sensitive). When yeast cells are grown in the presence of glucose, respiration is down-regulated, and Δψ is lowered because it is mainly generated by the ADP/ATP carrier, which shuttles ATP from the cytosol into the matrix space in exchange for ADP from the matrix. It was suggested that the TIM9-13 complex is only required for import of yeast Tim23 (yTim23) when the membrane potential Δψ is low (21).

To analyze whether DDP1 and hTim13 are able to substitute for the function of Tim8 and Tim13, we expressed the human components in the Δ8/Δ13 yeast mutant. Both proteins were detected in mitochondria and released from the intermembrane space upon disruption of the outer membrane using digitonin at a concentration of 0.05% (Fig. 4A).

Wild-type (WT), Δ8/Δ13, and Δ8/Δ13 yeast cells harboring DDP1 and hTim13 (Δ8/Δ13+DDP1/hTim13) were grown at 15 °C and 30 °C in the presence of glucose. The cells grew normally at 30 °C (Fig. 4, B and C, left panels). At 15 °C the growth rate of Δ8/Δ13 cells was reduced drastically compared with WT cells, whereas the Δ8/Δ13+DDP1/hTim13 cells grew nearly like WT (Fig. 4, B and C, right panels). Thus, DDP1 and hTim13 rescue the cold-sensitive deletion phenotype in yeast. This indicates that the human homologs are able to complement the function of the yeast TIM8-13 complex.

The DDP1-hTim13 Complex Facilitates Import of Yeast and Human Tim23—We have investigated the role of DDP1 and hTim13 in the import of mitochondrial precursor proteins into yeast mitochondria. Mitochondria isolated from WT, Δ8/Δ13, and Δ8/Δ13+DDP1/hTim13 yeast cells were either energized with NADH or preincubated with valinomycin and FCCP to dissipate the membrane potential. Radiolabeled precursor of yeast Tim23 and of human Tim23 was added, and import was measured. In the absence of Δψ, yTim23 accumulated at stage III, a translocation intermediate that is partially resistant to trypsin treatment (Fig. 5, upper left panel). In Δ8/Δ13 mitochondria the amount of the stage III intermediate was reduced to −50% of the WT level, indicating that the TIM8-13 complex is required to trap the translocation intermediate in the intermembrane space. In mitochondria from Δ8/Δ13+DDP1/hTim13 cells the formation of the stage III intermediate was restored to WT levels. This indicates that the DDP1-hTim13 complex interacts with the Tim23 precursor of yeast. When human Tim23 was incubated with the mitochondria in the absence of Δψ no significant amounts of trypsin-protected species were detected (Fig. 5, upper right panel). Similarly, no resistant intermediates were detected when proteinase K was used (not shown). Although stage III intermediates of hTim23 were detected by cross-linking (see below), it is apparently not possible to characterize them by protease protection assays.

In the presence of Δψ, yTim23 was equally well imported into yeast WT and Δ8/Δ13 mitochondria (Fig. 5, lower left panel), supporting the notion that the TIM8-13 complex is not strictly required for the biogenesis of yTim23 when the membrane potential is sufficiently strong (21). Yet, the import efficiency of yTim23 was increased significantly in mitochondria harboring the DDP1-hTim13 complex, demonstrating that the human or-
thologs contribute to import of the yeast precursor (Fig. 5, lower left panel). At high levels of $\Delta \psi$, $h$Tim23 was imported efficiently into WT mitochondria (Fig. 5, lower right panel). The import of $h$Tim23 was reduced significantly in mitochondria from $/\Delta \psi$ cells, suggesting that it is dependent on the TIM8-$\Delta \psi$ complex even at high $\Delta \psi$. When the human DDP1-$h$Tim13 complex was present the import efficiency of $h$Tim23 was restored. This indicates that the DDP1-$h$Tim13 complex facilitates translocation and insertion of $h$Tim23 into the mitochondrial inner membrane.

In conclusion, the DDP1-$h$Tim13 complex acts in the import of Tim23 and assists the translocation of the precursor across the outer membrane in a manner similar to the yeast TIM8-$\Delta \psi$ complex. In contrast to $y$Tim23, the import of $h$Tim23 is generally dependent on the assistance of the DDP1-$h$Tim13 complex even when the membrane potential is high.

The DDP1-$h$Tim13 Complex Interacts with the Stage III Translocation Intermediate of Human Tim23 in Mammalian Mitochondria—Import of hTim23 into isolated mouse liver mitochondria was dependent on $\Delta \psi$ (data not shown). To address whether the DDP1-$h$Tim13 complex is in contact with translocation intermediates (stage III) in mammalian mitochondria, we performed cross-linking. Mouse liver mitochondria were preincubated to dissipate $\Delta \psi$. Subsequently, radiolabeled hTim23 was added to accumulate stage III intermediates, and cross-linking with MBS was performed. Two major hTim23 cross-linking adducts were detected which correspond to apparent molecular masses of $\sim 33$ and $35$ kDa (Fig. 6A). To characterize these cross-linking products immunoprecipitations with antibodies against DDP1 and $h$Tim13.
FIG. 4. DDP1 and hTim13 are orthologs of yeast Tim8 and Tim13. A, expression of DDP1 and hTim13 in yeast mitochondria. Human cDNAs of DDP1 and hTim13 were expressed in yeast cells carrying a double deletion of yTim8 and yTim13 (Δ8/Δ13). Mitochondria from WT, Δ8/Δ13, and Δ8/Δ13 cells harboring the human components (Δ8/Δ13+DDP1/hTim13) were isolated. Equal amounts of mitochondrial protein were subjected to SDS-PAGE and analyzed by Western blotting using antibodies against the mitochondrial yeast proteins yTim23, yTim8, yTim13, and against DDP1 and hTim13. B and C, complementation of the cold-sensitive phenotype of Δ8/Δ13 in the presence of glucose. WT, Δα/Δ13, and Δ8/Δ13+DDP1/hTim13 strains were grown at 30 °C to an A600 of 1.0. B, the cultures were subjected to serial 10-fold dilutions, and 2-μl aliquots were spotted on YPD plates and incubated at 30 °C and 15 °C. C, the cultures were diluted to A600 = 0.05 in fresh YPD medium, and the liquid cultures were incubated at 15 °C and 30 °C. Once a day the cultures were rediluted to A600 = 0.05 in YPD medium prewarmed to the respective temperature. Aliquots were withdrawn after the indicated time periods, and the cell number was determined. The cell number at t = 0 h was set equal to 1. The experiments were repeated at least three times with similar results.

FIG. 5. The human DDP1/hTim13 complex facilitates the import of yeast and human Tim23 into yeast mitochondria. Radiolabeled hTim23 and yTim23 were synthesized in reticulocyte lysate and incubated for 15 min with mitochondria isolated from WT, Δ8/Δ13, and Δ8/Δ13+DDP1/hTim13 strain. The membrane potential was either dissipated with FCCP/valinomycin (upper panels; −Δψ) or strengthened with NADH (lower panels; +Δψ). Samples were treated with 50 μg/ml trypsin, analyzed by SDS-PAGE and Western blotting, and quantified using a PhosphorImaging system.

FIG. 6. The mammalian DDP1:hTim13 complex interacts with translocation intermediates of hTim23. Freshly isolated mouse liver mitochondria were pretreated with FCCP/valinomycin to dissipate the membrane potential. Radiolabeled human Tim23 precursor was added, and cross-linking with 100 μM MBS was performed where indicated (A). Mitochondria were solubilized with 0.5% Triton X-100, and immunoprecipitation with affinity-purified antibodies against DDP1 and hTim13 and preimmune IgG to DDP1 was carried out under non-denaturing conditions. Samples were subjected to SDS-PAGE, Western blotting, and autoradiography (B). hTim23-specific cross-link adducts (X-links) are indicated.

antibodies against DDP1 and hTim13 were performed (Fig. 6B). Under native conditions each of the antibodies precipitated both cross-linking products. This indicates that the hTim23 precursor interacts with the intact DDP1:hTim13 complex in the intermembrane space of mammalian mitochondria.

DISCUSSION

The Mohr-Tranebjaerg syndrome is considered to be the first mitochondrial disorder caused by a defect in the mitochondrial import machinery. The suggestion of the underlying pathomechanism is based on the homology of the dysfunctional DDP1 to a family of small yeast proteins that act along the TIM22-dependent import pathway. In yeast, this protein family comprises the essential proteins Tim9, Tim10, and Tim12, and the nonessential components Tim8 and Tim13 (8, 10, 11, 15, 19). They form three distinct heterooligomeric complexes in the intermembrane space which facilitate the transfer of hydrophobic precursors from the TOM complex to the TIM22 complex in the inner membrane. These "small" TIM complexes bind to the translocation intermediates during import thereby maintaining the precursors in an import-competent conformation (17, 21). The essential components differ from the nonessential components by their specificity for substrate preproteins. The TIM9-10 and the TIM9-10-12 complexes mediate the import of mitochondrial carrier proteins, whereas the TIM8-13 complex interacts specifically with translocation intermediates of Tim23, the major component of the inner membrane translocase for matrix targeted preproteins.

By analogy to the function of Tim8 and Tim13 in yeast it was suggested that the Mohr-Tranebjaerg syndrome is caused by a defect in the biogenesis of the human TIM23 complex (17, 21).
However, the TIM8:13 complex in yeast is not strictly required for the import of Tim23. A requirement of the TIM8:13 complex was only observed when membrane insertion of Tim23 was compromised (21). It was not clear whether the human DDP1 and hTim13 are true orthologs of Tim8 and Tim13 and fulfill a corresponding function in the import of Tim23.

In the present study, we analyzed the structural organization and function of DDP1 and hTim13. We show that both proteins are located in the intermembrane space. They are present in roughly equimolar amounts and form hetero-oligomeric assemblies with an apparent molecular mass of ~70 kDa. The 70-kDa complex appears to be composed of three molecules of DDP1 and three molecules of hTim13. However, it cannot be decided whether this stoichiometry is fixed or determined by the relative expression levels of the components.

DDP1 and hTim13 belong to a family of evolutionarily conserved proteins of the intermembrane space. In humans, six members have been identified all of which share four conserved cysteine residues (Cys4 motif) considered to constitute a metal binding site. We determined that DDP1 and hTim13 bind Zn$^{2+}$ ions in a 1:1 stoichiometry and thus appear to form zinc fingers. As in yeast these zinc fingers might be crucial for the recognition and binding of translocation intermediates by DDP1 and hTim13.

We demonstrated that the human DDP1-hTim13 complex is functional in yeast. It rescues the growth defect observed at low temperature in the yeast attim8/attim13 mutant, indicating that it is a true ortholog. Moreover, mitochondria from the complemented strain import yTim23 with a higher efficiency than WT yeast mitochondria. Thus, DDP1 and hTim13 so far are the only components of the mitochondrial inner membrane translocase that are functional across species. Considering the rather low sequence similarity between the human and yeast orthologs and its restriction to regions around the conserved cysteine pairs (18), the putative zinc finger might be the functionally important element of the DDP1-hTim13 complex.

In all but one patient with Mohr-Tranebjaerg syndrome this putative zinc finger is not expressed because of nonsense or frameshift mutations located upstream the Cys4 motif. In one patient a missense mutation, C66W, leads to a nonfunctional zinc finger. Studies on the mutant DDP1-C66W revealed that it does not accumulate in the intermembrane space of mitochondria from patient cell lines. This suggests that the mutant DDP1 protein is not able to fold properly and is degraded rapidly; this also explains the full-blown clinical phenotype observed in a patient harboring the mutant C66W allele on the X chromosome.

Has the DDP1-hTim13 complex a role in the biogenesis of the human TIM23 complex? Import of hTim23 into yeast mitochondria appears to be strongly dependent on the assistance of DDP1 and hTim13, whereas import of yTim23 does not require the yeast TIM8:13 complex under normal conditions. In particular, import of hTim23 into yeast mitochondria required the assistance of the DDP1-hTim13 complex even when the Δψ was high. Under these conditions biogenesis of yTim23 is independent of the yeast TIM8:13 complex. Furthermore, hTim23 was found to be in contact with the DDP1-hTim13 complex during import in isolated mammalian mitochondria. Together, these observations provide direct evidence that the DDP1-hTim13 complex fulfills an important role in import of Tim23 in mammals by trapping translocation intermediates in the intermembrane space (Fig. 7A). Mutations in DDP1 are likely to cause a defect in the biogenesis of the Tim23 in humans (Fig. 7B).

In summary, the DDP1-hTim13 complex acts in the intermembrane space and specifically assists the import of the Tim23 precursors into the mitochondrial inner membrane. The DDP1-hTim13 complex functions in a manner comparable to that of the yeast TIM8:13 complex. Whereas import of yeast Tim23 does not require the TIM8:13 complex under normal conditions, import of hTim23, by contrast, appears to rely on the assistance of the DDP1-hTim13 complex under all conditions. Accordingly, the pathomechanism underlying the Mohr-Tranebjaerg syndrome may therefore be based on a defective TIM23 translocase leading to severe pleitropic mitochondrial dysfunction.

Acknowledgments—We thank Bettina Treske for excellent technical assistance. We are grateful to Dr. P. Klužers and Helmhut Hartl for making possible the use of the ICP equipment, and Andreas Reichart for kindly providing HeLa cells.

REFERENCES
1. Ryan, K. R., and Jensen, R. E. (1995) Cell 83, 517–519
2. Tokatlidis, K., Junne, T., Moes, S., Schatz, G., Glick, B. S., and Kronidou, N. (1996) Nature 384, 585–588
3. Pfanner, N. (1998) Curr. Biol. 8, R262–R265
4. Herrmann, J. M., and Neupert, W. (2000) Curr. Opin. Microbiol. 3, 210–214
5. Bauer, M. F., Hofmann, I., Neupert, I., and Brunner, I. (2000) Trends Cell Biol. 10, 25–31
6. Berthold, J., Bauer, M. F., Schneider, H. C., Klaus, C., Dietmeier, K., Neupert, W., and Brunner, M. (1995) Cell 81, 1085–1093
7. Sirrenberg, C., Bauer, M. F., Guiard, B., Neupert, W., and Brunner, M. (1996) Nature 384, 582–585
8. Sirrenberg, C., Endres, M., Folsch, H., Stuart, R. A., Neupert, W., and Brunner, M. (1998) Nature 391, 912–915
9. Koehler, C., Holder, J., Schmitz, M., Jung, R. S., and Jensen, R. E. (1997) J. Cell Biol. 139, 1663–1675
10. Koehler, C. M., Merchant, S., Oppigliger, W., Schmid, K., Jarosch, E., Dolfini, L., Junne, T., Schatz, G., and Tokatlidis, K. (1998) EMBO J. 17, 6477–6486
11. Koehler, C. M., Jarosch, E., Tokatlidis, K., Schmid, K., Schweney, R. J., and Schatz, G. (1998) Science 279, 369–373
12. Bauer, M. F., Sirrenberg, C., Neupert, W., and Brunner, M. (1996) Cell 87, 33–41
13. Donzeau, M., Kaldi, K., Adam, A., Passen, S., Wanner, G., Guiard, B., Bauer, M. F., Neupert, W., and Brunner, M. (2000) Cell 101, 401–412
14. More, F., Sirrenberg, C., Schneider, H.-C., Neupert, W., and Brunner, M. (1999) EMBO J. 18, 3667–3675
15. Adam, A., Endres, M., Sirrenberg, C., Lottspeich, F., Neupert, W., and Brunner, M. (1999) EMBO J. 18, 313–319

C. Köhler, personal communication.
Role of Human DDP1 and Tim13 in the Biogenesis of Tim23

16. Endres, M., Neupert, W., and Brunner, M. (1999) EMBO J. 18, 3214–3221
17. Leuenberger, D., Bally, N. A., Schatz, G., and Koehler, C. M. (1999) EMBO J. 18, 4816–4822
18. Bauer, M. F., Rothbauer, U., Muhlenbein, N., Smith, R. J., Gerbitz, K., Neupert, W., Brunner, M., and Hofmann, S. (1999) FEBS Lett. 464, 41–47
19. Koehler, C. M., Leuenberger, D., Merchant, S., Renold, A., Junne, T., and Schatz, G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2141–2146
20. Jin, H., Kendall, E., Freeman, T. C., Roberts, R. G., and Vetrie, D. L. (1999) Genomics 61, 259–267
21. Paschen, S. A., Rothbauer, U., Kaldi, K., Bauer, M. F., Neupert, W., and Brunner, M. (2000) EMBO J. 19, 6392–6400
22. Davis, A. J., Sepuri, N. B., Holder, J., Johnson, A. E., and Jensen, R. E. (2000) J. Cell Biol. 150, 1271–1282
23. Tranebjaerg, L., Schwartz, C., Eriksen, H., Andreasson, S., Ponjavic, V., Dahl, A., Stevenson, R. E., May, M., Arena, F., and Barker, D. (1995) J. Med. Genet. 32, 257–263
24. Jin, H., May, M., Tranebjaerg, L., Kendall, E., Fontan, G., Jackson, J., Subramony, S. H., Arena, F., Lubs, H., Smith, S., Stevenson, R., Schwartz, C., and Vetrie, D. (1996) Nat. Genet. 14, 177–180
25. Tranebjaerg, L., Hamel, B. C., Gabreels, F. J., Renier, W. O., and Van Ghelue, M. (2000) Eur. J. Hum. Genet. 8, 464–467
26. Herrmann, J. M., Stuart, R. A., Craig, E. A., and Neupert, W. (1994) J. Cell Biol. 127, 893–902
27. Bauer, M. F., Gempel, K., Reichert, A. S., Rappold, G. A., Lichtner, P., Gerbitz, K. D., Neupert, W., Brunner, M., and Hofmann, S. (1999) J. Med. Biol. 289, 69–82
28. Schagger, H., Cramer, W. A., and von Jagow, G. (1994) Anal. Biochem. 217, 220–230
29. Schagger, H., and von Jagow, G. (1991) Anal. Biochem. 199, 223–31
30. Pelham, H. R., and Jackson, R. J. (1976) Eur. J. Biochem. 67, 247–256
Role of the Deafness Dystonia Peptide 1 (DDP1) in Import of Human Tim23 into the Inner Membrane of Mitochondria

Uli Rothbauer, Sabine Hofmann, Nicole Mühlenbein, Stefan A. Paschen, Klaus-Dieter Gerbitz, Walter Neupert, Michael Brunner and Matthias F. Bauer

J. Biol. Chem. 2001, 276:37327-37334. doi: 10.1074/jbc.M105313200 originally published online August 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105313200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 12 of which can be accessed free at http://www.jbc.org/content/276/40/37327.full.html#ref-list-1