The C(66)W Mutation in the Deafness-Dystonia Peptide 1 (DDP1) affects the formation of functional DDP1/TIM13 complexes in the mitochondrial intermembrane space

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Abstract

Mohr-Tranebjaerg syndrome is a progressive, neurodegenerative disorder caused by loss-of-function mutations in the DDP1/TIMM8A gene. DDP1 belongs to a family of evolutionary conserved proteins which are organized in hetero-oligomeric complexes in the mitochondrial intermembrane space. They mediate the import and insertion of hydrophobic membrane proteins into the mitochondrial inner membrane. All of them share a conserved Cys4 metal binding site proposed to be required for the formation of zinc fingers. So far, the only missense mutation (C66W) known to cause a full-blown clinical phenotype directly affects this Cys4 motif. Here, we show that the mutant human protein is efficiently imported into mitochondria and sorted into the intermembrane space. In contrast to wild-type DDP1 it does not complement the function of its yeast homologue Tim8. The C66W mutation impairs binding of Zn2+ ions via the Cys4 motif. As a consequence, the mutated DDP1 is incorrectly folded and loses its ability to assemble into a hetero-hexameric 70 kDa complex with its cognate partner protein human Tim13. Thus, an assembly defect of DDP1 is the molecular basis of Mohr-Tranebjaerg syndrome in patients carrying the C66W mutation.
Introduction

With the exception of a few components of the oxidative phosphorylation machinery, all mitochondrial proteins are encoded by nuclear genes and synthesized on cytosolic ribosomes. The import of such preproteins into mitochondria and the correct sorting into mitochondrial subcompartments is mediated by a set of import systems in the outer and inner mitochondrial membrane. Three distinct preprotein import systems have been described (1-5). All preproteins most likely use the general translocase in the outer membrane, the TOM complex. It mediates the recognition and binding of preproteins as well as their transfer across the outer membrane. Further movement of the translocation intermediates into and across the inner membrane is mediated by two distinct translocases in the inner membrane, the TIM23 and the TIM22 complexes. The TIM23 complex mediates import of preproteins with positively charged targeting signals at their N-termini into the mitochondrial matrix space and into the inner membrane (6-8). The transfer of preproteins across the inner membrane strictly requires both an electrochemical potential ($\Delta \psi$) across the inner membrane and ATP in the matrix as energy sources (9). The TIM22 complex is used by a class of hydrophobic inner membrane proteins with internal and so far less characterized targeting signals (10-15). Typical substrates are the members of the mitochondrial carrier family that are synthesized without a matrix targeting signal. In addition, the TIM22 complex appears to mediate the import of precursors of other hydrophobic membrane proteins such as Tim23 and Tim22, which do not belong to the class of mitochondrial carriers (16-18). Insertion of these precursors into the inner membrane is strictly dependent on $\Delta \psi$ but does not require ATP in the matrix.

Proteins destined for the inner membrane require the help of small, structurally related Tim proteins in the intermembrane space. In the yeast *S. cerevisiae*, five members of this protein
family are expressed (19). Of these, Tim9, Tim10 and Tim12 specifically support the import of mitochondrial carrier preproteins. (11-14,16,20). They form two distinct hetero-oligomeric complexes of 70 kDa which interact with the hydrophobic precursors thereby keeping them in an import competent conformation. The TIM9-10 complex interacts with the carrier proteins early in the import pathway when they are partially translocated across the TOM complex. The carrier proteins are then handed over to the TIM9-10-12 complex which is tightly associated with the TIM22 complex and mediates their insertion into the inner membrane.

Tim8 and Tim13 assist the import of a distinct subgroup of inner membrane proteins such as Tim23, the major component of the TIM23 translocase (16,21-23). Like the other members of the family they exist as hetero-oligomeric 70 kDa complexes in the intermembrane space but, in contrast to Tim9, Tim10 and Tim12, are not essential for the cell viability in yeast. The TIM8-13 complex binds to the incoming Tim23 precursor still associated with the TOM complex. In yeast, it is only required when the membrane potential is low and the membrane insertion of Tim23 is inefficient (21). Under these conditions the TIM8-13 complex is necessary to accumulate the Tim23 precursor and present it to the TIM22 complex thereby facilitating its insertion into the inner membrane.

The human homologue of Tim8 is encoded by the DDP1 (deafness dystonia peptide 1) gene. Mutations in the DDP1/TIMM8A gene cause the Mohr-Tranebjaerg syndrome, a progressive, neurodegenerative disorder characterized by sensorineural hearing loss, dystonia, mental retardation and blindness (24,25). Most of the patients harbor loss-of-function mutations leading to a complete absence of the DDP1 protein. In human mitochondria, DDP1 forms a hetero-oligomeric complex of 70 kDa together with hTim13 (26). The DDP1-hTim13 complex specifically assists the import of the human Tim23 precursors into the inner
membrane (26). The human complex is able to complement the function of the TIM8-13 complex in yeast. Whereas import of yeast Tim23 does not require the TIM8-13 complex under normal conditions, import of human Tim23 appear to be dependent on the assistance of the DDP1-hTim13 complex under all conditions studied (26). It was therefore suggested that the pathomechanism underlying Mohr-Tranebjaerg syndrome may involve an impaired biogenesis of the human TIM23 complex.

The small Tim proteins belong to an evolutionary conserved protein family characterized by a common Cys4 metal binding motif. Binding of Zn^{2+} ions was proposed to be required for the formation of typical zinc finger structures (13,19,26). These zinc fingers may be crucial for the recognition and binding of translocation intermediates during their transfer through the aqueous environment of the intermembrane space.

In this report, we analyzed the structural and functional consequences of a mutation (C66W), directly affecting the Cys4 metal binding motif. This cysteine to tryptophan exchange at amino acid position 66 is currently the only missense mutation known to cause Mohr-Tranebjaerg syndrome (27). We show that the DDP1^{C66W} is efficiently imported into mitochondria and correctly sorted into the intermembrane space. However, the mutant protein is no more able to complement the function of Tim8 in yeast mitochondria lacking the endogenous TIM8-13 complex; in particular, the mutant DDP1 cannot restore import of Tim23. Analysis of purified recombinant DDP1 revealed that the C66W exchange impairs the ability of DDP1 to bind zinc. As a consequence, DDP1^{C66W} does not fold properly and is no longer able to interact with its partner protein hTim13. Thus, the C66W substitution leads to a defect in the formation of a functional DDP1-hTim13 complex. This may interfere with the biogenesis of the TIM23 complex and an impaired biogenesis of the mitochondria might by
the pathomechanistic basis of the Mohr-Tranebjaerg syndrome.

**Materials and Methods**

*Plasmids and strain construction* - For subcloning of DDP1 and hTim13 into expression vectors, DNA was PCR-amplified from DDP1- and hTim13-specific EST clones (19) using specific oligonucleotide primers (DDP1-F: 5'-atg gat tcc tcc tct tcc tcc-3'; DDP1-R: 5'-tca gtc aga aag gct ttc tga g-3'; hTim13-F: atg gag ggc ggc ttc gg-3'; hTim13-R: 5'-tca cat gtt ggc teg ttc-3'). DDP1 and hTim13 were subcloned into the EcoRI/HindIII sites of yeast expression vectors pYX232(TRP1) and pYX142(LEU2) yeast expression vectors (Novagen), respectively, as described before (26). Overlap extension PCR was performed to construct the DDP1_C66W using mutagenic primers 5'-ctc aac cca gtt cac aaa a-3' and 5'-ttg tga act tgg cgg ttg agc-3' and flanking primers DDP1-F and DDP1-R. Bold underlined letters indicate the altered nucleotide. The mutation was verified by direct sequencing of the resulting PCR fragment. The fragment was then cloned into the EcoRI/HindIII site of vector pYX232(TRP1). The *Saccharomyces cerevisiae* strain MB2-Δ8Δ13 (*tim8::HIS3; tim13::URA3*) (21) was transformed with either the DDP1- or DDP1_C66W plasmid together with the hTim13 plasmid and transformants were selected on minimal medium lacking histidine, uracil, leucine and tryptophan. Maltose binding protein (MBP) fusion proteins were generated by subcloning DDP1, DDP1_C66W or hTim13 into the BamHI/XbaI site of pMal-cRI vectors (New England Biolabs). His-tagged versions of DDP1 and DDP1_C66W were generated by subcloning PCR-amplified fragments into the BamHI/HindIII site of pQE40 (Qiagen). For construction of the chimeric hTim13-glutationine S-transferase (GST) protein, hTim13 was PCR-amplified using primers 5'-ccc gaa ttc att aaa gag gag aaa tta act atg gag ggc ggc ttc gg -3' / 5'-ggg ccc ggg aca tgt tgg ctc gtt ccc gc-3' and digested using restriction enzymes
EcoRI and SmaI. The digested hTim13 fragment was subcloned into a EcoRI/Smal digested pQE13-Tim23(1-101)GST vector thereby removing the his6-tag from the plasmid (7). For in vitro synthesis of precursor proteins, human Tim23, DDP1 and DDP1C66W fragments were digested with EcoRI/HindIII and subcloned into pGEM4.

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 (ICN). Import reactions into isolated yeast mitochondria or mitochondria prepared from mouse liver were carried out for 10 - 15 min at 25°C in 180 µl of 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) in the presence of 2.5 mM ATP and 5 mM NADH. To energize mitochondria from mouse liver 2.5 mM succinate was added. The reactions contained 180 µg of mitochondrial protein and 4 % reticulocyte lysate with the radiolabeled precursor proteins. Membrane potential was dissipated, when indicated, by omission of NADH and pre-incubation for 5 min at 25°C with 1 µM valinomycin and 25 µM FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone). Aliquots (50 µl) each corresponding to 50 µg of mitochondrial protein were either incubated with proteinase K for 20 min on ice followed by incubation with protease inhibitor (5 min on ice) or left untreated. Mitochondria were washed twice in HS buffer (20 mM Hepes-KOH pH 7.4, 0.6 M sorbitol), subjected to SDS-PAGE and blotted onto nitrocellulose membranes. The amount of precursor was quantified using a Fuji PhosphoImaging system.

To analyze the stability of DDP1C66W precursors, radiolabeled precursor was imported into isolated mouse mitochondria. The reactions contained 300 µg of mitochondrial protein and
4% reticulocyte lysate. The import was stopped after 10 min by incubation with trypsin (100 µg/ml) for 20 min on ice followed by incubation with soybean trypsin inhibitor (500 µg/ml) for 5 min. Mitochondria were washed twice in HS buffer and resuspended in import buffer supplemented with 2.5 mM ATP, 5 mM NADH, 0.1 mg/ml creatine kinase and 10 mM creatine phosphate. Aliquots corresponding to 50 µg mitochondria were removed after 5, 10, 30, 60 and 90 min, subjected to SDS-PAGE and blotted onto nitrocellulose.

Determination of zinc - MBP-DDP1, MBP-DDP1C66W and MBP-lacα fusion proteins were expressed in XL1-blue *Escherichia 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 7.4, 100 mM NaCl, 20 mM β-mercaptoethanol. A clarifying spin was performed and the supernatant was directly applied to an amylose column for affinity purification. After binding the column was washed with 50 ml lysis buffer lacking the zinc acetate. Elution was performed 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.

Analysis of recombinant proteins - *E. coli* strain XL-1 blue was transformed with pQE-expression plasmids harboring either his-tagged versions of DDP1 and DDP1C66W or untagged hTim13-GST. Cultures were grown to an OD₅₇₈ of 0.5 prior to induction with 1mM IPTG for 4 h. The cells were harvested by centrifugation, resuspended in lysis buffer (1 x PBS, pH 7.4, 1 mM PMSF) and lysed by addition of lysozyme (100 µg/ml) and repeated sonication. The cell lysates were centrifuged at 10,000 g at 4°C for 20 min. *E. coli* supernatant containing either hisDDP1 or hisDDP1C66W were loaded onto Ni-NTA columns (Qiagen). After washing, bound material was eluted with 300 mM imidazole. Elution fractions
containing his-tagged proteins were pooled and desalted using a PD-10 column (Amersham Pharmacia). To determine the sensitivity of the recombinant proteins to trypsin, aliquots (2 µg each) of purified hisDDP1 or hisDDP1C66W were diluted in PBS and incubated with increasing concentrations of trypsin for 10 min at 0°C. The samples were TCA-precipitated and analyzed by immunoblot analyses using a Penta-His antibody (Qiagen). For crosslinking experiments, a 2 µg aliquot of purified recombinant protein was diluted in 20 mM Hepes-KOH buffer (pH 7.4) and crosslinker was added to a final concentration of 100 µM. After incubation for 30 min at room temperature the crosslinking reactions were quenched with 80 mM Tris/HCl, pH 8.0. TCA-precipitated crosslinking adducts were analyzed by immunoblotting using a Penta-His antibody.

To determine the oligomeric state of recombinant proteins, 5 µg of purified and desalted hisDDP1 or hisDDP1C66W were subjected to a Superdex-75 gel filtration column (Amersham Pharmacia) and chromatographed at a flow-rate of 0.2 ml/min in column buffer containing 1 x PBS, pH 7.4 and 10 % glycerol. Fractions (0.5 ml) were analyzed by SDS-PAGE and immunoblotting with antibodies against Penta-His. As calibration standards bovine serum albumin, carbonic anhydrase and cytochrome c were used.

In order to analyze the interaction between DDP1 and hTim13 in vitro, recombinant hisDDP1 or hisDDP1C66W was bound to Ni-NTA resin and washed with 10 volumes of buffer (1 x PBS, pH 7.4, 20 mM Imidazol). Subsequently, *E. coli* suspensions expressing hTim13-GST were lysed and centrifuged at 10,000 g (20 min at 4°C). The supernatant was loaded onto the prebound columns. After an over-night incubation, the columns were extensively washed with washing buffer to remove unspecifically bound material. His-tagged proteins were then eluted using 5 volumes of equilibration buffer containing 300 mM imidazole. Aliquots (30 µl) of the elution fractions were removed for direct analysis. In a second purification step, elution
fractions were incubated with glutathione sepharose beads (GSH beads). Material bound to the beads was released in Laemmli buffer, applied to SDS-PAGE and analyzed by Western blotting using affinity purified antibodies against DDP1 and hTim13.

Miscellaneous - Mitochondria from yeast were isolated as described (28). Mitochondria from mouse liver were prepared in a medium containing 0.25 M sucrose, 5 mM EDTA, 20 mM Tris/HCl pH 7.4 by differential centrifugation and subsequent sucrose step gradient ultracentrifugation as described previously (29). Antisera against the C-termini of DDP1, hTim13 and hTim23 were raised in rabbits by injecting the chemically synthesized peptides as described before (26,29). RNA was prepared from 10 ml liquid yeast cultures grown to an OD1.0. Cells were resuspended in AE buffer (50 mM Na-acetate, 10 mM EDTA, pH 5.3). SDS was added to a final concentration of 1 % and RNA was isolated using a phenol/freeze protocol. Ethanol precipitated total RNA was used for first-strand cDNA synthesis using Superscript II reverse transcriptase (Gibco) and oligo d(T) primers.

Results

The C66W exchange impairs the function of DDP1 in yeast - A patient with Mohr-Tranebjaerg syndrome was recently reported to carry a mutation in DDP1 leading to an exchange of cysteine to tryptophan at amino acid position 66 (DDP1C66W) (27). To functionally characterize the C66W substitution, we expressed the mutant human DDP1C66W protein in yeast cells carrying disrupted alleles of TIM8 and TIM13 (Δ8/Δ13) (21). Tim8 and Tim13 are the yeast homologues of DDP1 and hTim13. They are not essential for cell viability. However, growth of Δ8/Δ13 at 15°C is severely reduced in the presence of glucose as a carbon source (21,26). Co-expression of human DDP1 and hTim13 in the Δ8/Δ13 strain
(Δ8/Δ13+DDP1/hTim13) results in a wild-type-like growth at 15°C indicating that the human components are able to complement the function of yeast Tim8 and Tim13 (Fig. 1A and B). Rescue of the cold-sensitive phenotype represents a model system to functionally analyze pathogenic DDP1 mutations.

Therefore, the C66W exchange was introduced by PCR-based mutagenesis and DDP1C66W was expressed in Δ8/Δ13 cells together with hTim13 (Δ8/Δ13+C66W/hTim13). When the transformed cells were grown at 15°C in the presence of glucose the mutant DDP1C66W was not able to rescue the cold-sensitive phenotype of Δ8/Δ13 (Fig. 1A and B). This indicates that a mutation affecting the putative zinc finger of DDP1 leads to a non-functional protein.

As shown previously the import efficiency of yeast and human Tim23 precursors in vitro is reduced significantly in mitochondria from Δ8/Δ13 cells (21,26). While import of yeast Tim23 is affected in mitochondria from Δ8/Δ13 cells only under conditions of low membrane potential, import of human Tim23 is reduced in Δ8/Δ13 mitochondria under all conditions. When DDP1 and hTim13 are expressed together in Δ8/Δ13 cells, the import of yeast and human Tim23 precursors is restored to wild type levels (26). We therefore investigated whether the mutant DDP1 is still able to restore the import of Tim23. Figure 2 shows the comparison of human Tim23 import into energized mitochondria from Δ8/Δ13+DDP1/hTim13 and Δ8/Δ13+C66W/hTim13 yeast cells. Import efficiency was decreased by about 75 % in mitochondria expressing DDP1C66W instead of wild-type DDP1. Thus, DDP1C66W is non-functional in yeast. Compared to wild-type DDP1, mutant DDP1C66W is not able to restore the import of Tim23 precursor into Δ8/Δ13 mitochondria. This dysfunction of DDP1C66W could result from impaired import into mitochondria, protein instability or from impaired complex formation.
**DDP1C66W is expressed in the mitochondrial intermembrane space** - To assess whether DDP1C66W was expressed in yeast, RNA was prepared from Δ8/Δ13 strains harboring plasmids encoding hTim13 and either DDP1 or DDP1C66W and analyzed by RT-PCR (Fig. 3A). DDP1C66W and DDP1 specific probes were amplified, indicating that both were expressed.

Mitochondria were prepared from the strains Δ8/Δ13+DDP1/hTim13 and Δ8/Δ13+C66W/hTim13, and analyzed by immunoblotting. Both, DDP1 and DDP1C66W were detected in the yeast mitochondria (Fig. 3B). The levels of DDP1C66W appear to be only slightly lower as compared to those of wild type DDP1. DDP1C66W was resistant to proteinase K treatment and became accessible to protease upon swelling of mitochondria, in a manner similar to the wild-type DDP1 (data not shown). Thus, DDP1C66W accumulated in the intermembrane space of yeast mitochondria. Similarly, hTim13 could be detected in mitochondria of the yeast strains expressing wild-type or mutant DDP1. However, the level of hTim13 appeared to be reduced to about 50% in the presence of DDP1C66W compared to yeast expressing wild type DDP1 (Fig. 3B).

To investigate whether the C66W substitution affects import of the mutant protein into the intermembrane space, DDP1 and DDP1C66W were synthesized in reticulocyte lysates in the presence of [35S]methionine. The radiolabeled proteins were then incubated with isolated yeast mitochondria. To assess translocation of radiolabeled precursors across the outer mitochondrial membrane the mitochondria were treated with proteinase K (PK). DDP1 and DDP1C66W were protected from proteinase K digestion. Both proteins were, however, degraded when mitoplasts were generated and treated with PK (Fig. 4A, left panels). This
indicates that DDP1 and the mutant protein DDP1C66W were efficiently imported into the intermembrane space of mitochondria. Both DDP1 and DDP1C66W precursors were also equally well imported into mitochondria isolated from mouse liver (Fig. 4A, right panels). Import of wild type and mutant DDP1 into the intermembrane space was not dependent on a membrane potential across the inner membrane (Fig. 4B). Thus, the C66W exchange does not impair import of DDP1 across the mitochondrial outer membrane and its correct sorting into the mitochondrial intermembrane space.

To assess the stability of the imported precursors in the intermembrane space, the kinetics of degradation of DDP1 and DDP1C66W were compared. Therefore, radiolabeled DDP1 or DDP1C66W precursor was imported into mitochondria isolated from mouse liver and import was stopped after 10 min by treatment with trypsin to remove non-imported precursor. Mitochondria were re-isolated and incubated at 37°C in the presence of ATP and an ATP-regenerating system (CK/Cr-P system) to stimulate degradation of proteins in the intermembrane space by the i-AAA protease Yme1p (30). The DDP1 level was reduced by 20% within 90 min, while the amount of DDP1C66W decreased by about 55% within the same time period (Fig. 5) suggesting that the turn-over rate of the mutant DDP1C66W was significantly higher than that of wild-type DDP1.

The C66W missense mutation impairs binding of zinc via the Cys4 motif – Cysteine-66 is part of a Cys4 zinc binding motif which is conserved in the family of small Tim proteins (19). To analyze whether the C66W exchange affects zinc binding properties of DDP1 we constructed chimeric proteins consisting of DDP1 or DDP1C66W fused to the C-terminus of MBP. For control, a MBP fused to the α-fragment of the β-galactosidase (MBP-α) was
used. The recombinant proteins were expressed in \textit{E. coli} in the presence of 1 mM Zn$^{2+}$, affinity purified on amylose resin and analyzed using ICP. The atomic emission was determined at the zinc-specific wavelength of 213.857 nm and the Zn$^{2+}$ content was calculated. The preparation of MBP-DDP1 contained about 1 mol Zn$^{2+}$ ion per mol fusion protein (Fig. 6A and B). In contrast, the preparation of MBP-DDP1C66W contained only background levels of zinc, comparable to zinc levels obtained with the control preparation of MBP-\(\alpha\) (Fig. 6A and B). Thus, the C66W exchange impairs zinc binding of DDP1.

\textit{Binding of Zn$^{2+}$ ions is required for stable folding of DDP1} - We tested whether zinc binding is required to stabilize the folded conformation of DDP1. His-tagged versions of DDP1 or DDP1C66W were expressed in \textit{E. coli} and purified by Ni-NTA chromatography. Aliquots (corresponding to 2 \(\mu\)g protein) were subjected to digestion with increasing amounts of trypsin (Fig. 7). DDP1 was clipped to a fragment of lower molecular weight which was resistant to protease treatment up to a concentration of 100 \(\mu\)g/ml of trypsin. In contrast, when DDP1 was pre-incubated with the metal-chelating agents EDTA and o-phenantroline (EDTA/o-phe) it was completely degraded by 10 \(\mu\)g/ml trypsin (Fig. 7). This indicates that zinc binding stabilizes the folded conformation of DDP1.

HisDDP1C66W was sensitive to treatment with lower concentrations of trypsin in the absence of metal chelating reagents (Fig. 7) indicating that the mutant protein was loosely or improperly folded. Since hisDDP1C66W does not bind zinc, the data demonstrate that the C66W substitution impairs stable folding of DDP1 by affecting the Cys$_4$ metal binding motif.

\textit{C66W mutation affects the oligomeric state of recombinant DDP1} - His-tagged versions of
DDP1 and DDP1C66W were expressed in *E. coli* and purified by Ni-NTA chromatography. The recombinant proteins were subjected to gel filtration analysis in order to assess their oligomeric state. HisDDP1 eluted in a fraction corresponding to a molecular mass of 65 kDa, indicating that the recombinant protein assembles into homo-oligomeric complexes (Fig. 8A and B). HisDDP1C66W eluted predominantly in the void volume of the Superdex-75 column suggesting that the mutant form of DDP1 was aggregated (not shown). A small portion of hisDDP1C66W was found in the 65 kDa fraction. This suggests that the C66W exchange impairs the ability of DDP1 to form homo-oligomers.

For further analysis, the recombinant proteins were subjected to chemical crosslinking with the aminospecific crosslinker disuccinimidyl suberate (DSS). The crosslinked adducts were analyzed by SDS-PAGE and Western blotting. In the absence of DSS, the electrophoretic mobility of hisDDP1 corresponded to a monomer (Fig. 8C). A small fraction (<3 %) of hisDDP1 migrated corresponding to a dimer which could not be cleaved by addition of DTT or β-mercaptoethanol (data not shown). This dimeric form may, therefore, represent a spontaneous crosslinking product. In the presence of DSS, adducts corresponding to dimeric, trimeric, tetrameric, pentameric and hexameric hisDDP1 were detected (Fig. 8C, arrows). No crosslinking products of higher oligomeric state were generated even when higher concentrations of DSS were used or when crosslinking was performed with glutaraldehyde, a less specific crosslinking reagent (data not shown). This supports that purified hisDDP1 can form homo-hexameric complexes *in vitro*. *In vivo*, DDP1 assembles into a hetero-oligomeric complex with its structurally related partner protein hTim13 (26).

HisDDP1C66W migrated predominantly in a monomeric form in the absence of DSS (Fig. 8C). Like hisDDP1, a small fraction of hisDDP1C66W was unspecifically crosslinked to a
Formation of a zinc finger structure is required for the interaction between DDP1 and hTim13 – In human mitochondria DDP1 and hTim13 assemble into hetero-oligomeric complexes in the intermembrane space (26). In order to analyze whether the C66W mutation also affects the association of DDP1 and hTim13 we performed a two-step interaction assay in vitro. In the first step hisDDP1 or hisDDP1C66W were bound to Ni-NTA sepharose. Subsequently, the hisDDP1 or hisDDP1C66W affinity resins were incubated with E. coli lysates containing hTim13-GST, a chimeric fusion protein of human Tim13 and glutathione S-transferase. After extensive washing steps the bound material was eluted with imidazol and aliquots were analyzed. hTim13-GST co-eluted together with hisDDP1, whereas only traces of hTim13-GST were detected in the peak elution fraction together with hisDDP1C66W (Fig. 9A and B, left panels). To exclude non-specific binding of GST fusion proteins to the Ni-NTA resin, the eluates were applied to a second purification step using glutathione sepharose beads (GSH beads). The GSH beads were extensively washed. Bound material was released using Laemmlli buffer and analyzed by SDS-PAGE and Western blotting. The bound material originating from the hisDDP1 column contained hisDDP1 and hTim13-GST indicating that both proteins directly interact in vitro (Fig. 9A, middle panel). As estimated from Coomassie blue stained gels hisDDP1 and hTim13-GST were present in roughly stoichiometric amounts in these complexes (Fig. 9A, right panel). In contrast, no hisDDP1C66W was detected after the second purification step using GSH beads (Fig. 9B, right panel). Thus, the C66W

dimer. In the presence of DSS the amount of the dimeric hisDDP1C66W increased slightly, however, no adducts of higher oligomeric state were formed, supporting the conclusion that the C66W substitution affects the complex formation of the mutant protein.
exchange impairs the direct interaction between DDP1 and its cognate partner protein hTim13 thereby compromising the ability to form functional hetero-oligomeric complexes.

**Discussion**

Deletion of the DDP1/TIMM8A gene or loss-of-functions mutation within the gene are associated with a severe disorder in humans, the Mohr-Tranebjaerg syndrome (OMIM # 304700). Despite some variability in its phenotypic expression, Mohr-Tranebjaerg syndrome is characterized by sensorineural hearing loss, dystonia, mental deterioration and optic atrophy, indicating progressive neurodegeneration. Recently, a first case of Mohr-Tranebjaerg syndrome with a missense mutation in DDP1/TIMM8A was reported (27). The mutated gene encodes a full length protein; however, the clinical outcome of this patient is indistinguishable from published clinical courses of patients with loss-of-function mutations leading to absent or truncated DDP1 proteins. The missense mutation is an amino acid exchange from cysteine to tryptophan (C66W) affecting one of the four highly conserved cysteine residues which constitute a Cys4 zinc finger motif (13,26). The precise functional role of the putative zinc fingers is currently unknown. They may be involved in folding or stability of the protein, in complex assembly or substrate recognition.

In the present study, we generated DDP1 constructs carrying the C66W mutation in order to analyze which function of DDP1 is affected. We combined *in vitro* analysis of purified recombinant protein with *in vivo* functional analysis of the mutant DDP1 in the yeast system. The Δ8/Δ13 yeast strain, lacking both yTim8 and yTim13, enabled us to test the function of the mutated DDP1. As demonstrated previously, expression of wild-type DDP1 together with human Tim13 in Δ8/Δ13 cells rescues the cold sensitive phenotype of the Δ8/Δ13 yeast strain (26). In contrast, when DDP1C66W instead of wild-type DDP1 was introduced into Δ8/Δ13
cells, transformants failed to grow on glucose at 15°C indicating that the mutant DDP1 is non-functional in yeast. Furthermore, mutant DDP1 is apparently no more able to take over the function of its yeast homologue Tim8 during import of Tim23. We showed that the DDP1C66W precursor is efficiently imported into mitochondria and correctly sorted into the intermembrane space. Although the mutant DDP1 appears to be about 2-fold more susceptible to degradation by endogeneous proteases, it accumulates in mitochondria to a level near that of wild-type DDP1. Thus, the inability of the mutant DDP1 to complement strain Δ8/Δ13 does not result from a reduced protein level in mitochondria.

The C66W mutation affects a highly conserved element within DDP1 consisting of four cysteines which likely form a metal binding site. We recently demonstrated that DDP1 and also its partner protein hTim13 indeed bind zinc in a 1:1 stoichiometry and thus appear to form zinc fingers (26). We have shown that the C66W mutation impairs the ability of DDP1 to bind zinc ions. As a consequence, the C66W mutation leads to an incorrectly folded protein, as indicated by its increased susceptibility to externally added protease. Likewise, pretreatment of wild-type DDP1 with the metal-chelating agents EDTA/o-phe results in an increased sensitivity of the protein to trypsin. It is therefore likely, that the C66W mutation alters the three-dimensional structure of the zinc binding domain thereby influencing the folding properties of DDP1.

What are the functional consequences of an altered zinc finger structure? Zinc fingers are ubiquitous structural elements which are associated with protein-nucleic acid recognition or protein-protein interactions (31). Our data provide evidence that a functional zinc finger is required for assembly of DDP1 into the 70 kDa complexes. Experiments in vitro using recombinant DDP1 and hTim13 showed that the interaction between these cognate partner proteins is completely abolished by the C66W mutation. Moreover, recombinant DDP1C66W
had lost the ability to form higher molecular weight complexes. In contrast, purified wild-type DDP1 or hTim13 spontaneously assembled into hexameric complexes even in the absence of the cognate partner protein. Thus, the structural features of the zinc fingers are conserved between DDP1 and hTim13 allowing association into homo- or hetero-oligomeric complexes. In vivo, both partner proteins are present in comparable concentrations and assemble into hetero-oligomers (26).

Currently, it is unclear whether the zinc finger in DDP1 is also involved in substrate recognition and binding during import of human Tim23. The DDP1C66W construct, however, is not suitable to answer this question since it can not be excluded that a loss of substrate binding is secondary to its inability to form a complex with hTim13. Thus, in order to identify the substrate binding sites of DDP1, the generation of further mutant DDP1 constructs is needed which harbor an intact Cys4 metal motif and are able to interact with hTim13.

In summary, we showed that exchange of one of the conserved cysteine residues leads to impaired zinc binding and subsequent incorrect folding of the zinc finger domain. The functional consequence of the C66W mutation is primarily a deficiency of DDP1 to form a 70 kDa complex together with hTim13 in the mitochondrial intermembrane space. Thus, although the mutated DDP1 protein is imported into mitochondria and correctly sorted, it is non-functional and leads to a full-blown clinical phenotype in Mohr-Tranebjaerg patients.

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Footnotes

The abbreviations used are: TOM, translocase of the mitochondrial outer membrane; TIM, translocase of the mitochondrial inner membrane; \( \Delta \psi \), membrane potential; DDP1, deafness dystonia peptide 1; PCR, polymerase chain reaction; EST, expressed sequence tags; MBP, maltose binding protein; GST, glutathione S-transferase; FCCP, carbonyl cyanide-\( p\)-trifluoromethoxyphenylhydrazone; PAGE, polyacrylamide gel electrophoresis; ICP, inductively coupled plasma atomic emission spectroscopy; PBS, phosphate-buffered saline; DSS, disuccinimidyl suberate.
**References**

**Figure legends**

**Figure 1**

**DDP1C66W does not complement the function of yeast Tim8.** Yeast cells carrying a double deletion of yTim8 and yTim13 (Δ8/Δ13) were transformed with plasmids expressing either DDP1 and hTim13 (Δ8/Δ13+DDP1/hTim13) or DDP1C66W and hTim13 (Δ8/Δ13+C66W/hTim13). A, WT, Δ8/Δ13, Δ8/Δ13+DDP1/hTim13 and Δ8/Δ13+C66W/hTim13 strains were grown at 30°C to an A578 of 1.0. The cultures were subjected to serial 10-fold dilutions, and 2-µl aliquots were spotted on YPD plates and incubated at 15°C to induce the cold-sensitive phenotype of the mutant Δ8/Δ13 strain in the presence of glucose. B, the cultures were diluted to A578 = 0.05 in YPD medium, and the liquid cultures were incubated at 15°C. The cultures were rediluted every 24 hours to A578 = 0.05 in YPD. 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.

**Figure 2**

**Import of human Tim23 precursor into Δ8/Δ13 cells expressing either wild-type or mutant DDP1.** Radiolabeled human Tim23 precursor was synthesized in reticulocyte lysate and incubated at 25°C for 10 min with energized mitochondria isolated from Δ8/Δ13+DDP1/hTim13 and Δ8/Δ13+C66W1/hTim13 yeast cells. After import, samples were treated with 50 µg/ml proteinase K (PK) to remove non-imported precursor and analyzed by SDS-
PAGE and autoradiography.

Figure 3

**Expression of the DDP1C66W protein in yeast mitochondria.** A, Detection of DDP1 and DDP1C66W specific transcripts by RT-PCR. DDP1 and DDP1C66W transcripts were amplified from first-strand cDNA prepared from Δ8/Δ13, Δ8/Δ13+DDP1/hTim13 and Δ8/Δ13+C66W/hTim13 yeast cells. A 294 base pair fragment could be detected in preparations from both transformed strains but not in the control strain Δ8/Δ13. B, Localization of the DDP1C66W to the mitochondrial intermembrane space. Mitochondria were prepared from Δ8/Δ13+DDP1/hTim13 and Δ8/Δ13+C66W/hTim13 yeast cells. Aliquots corresponding to 50 µg mitochondrial protein were separated by SDS-PAGE and analyzed by immunoblotting using affinity purified antibodies against DDP1 and hTim13.

Figure 4

**Import of DDP1 and DDP1C66W into mitochondria.** A, Radiolabeled precursors of DDP1 or DDP1C66W were synthesized in reticulocyte lysate in the presence of [35S]methionine and imported for 15 min at 25°C into energized mitochondria isolated from yeast cells (left panels) or from mouse liver (right panels). Aliquots each corresponding to 50 µg mitochondrial protein were either incubated with 100 µg/ml proteinase K (PK) to remove non-imported precursor or left untreated. When indicated, mitoplasts were generated by hypotonic swelling (SW) or by solubilization with 0.1% digitonin (Dig) and then treated with protease. Samples were analyzed by SDS-PAGE and autoradiography. To confirm complete opening of the outer membrane by swelling or digitonin solubilization, Western blotting using antibodies
against the endogeneous yeast Tim13 (left panel) or the endogeneous mouse DDP1 (right panel) was performed. B, Import of DDP1 and DDP1C66W does not require a membrane potential Δψ. Radiolabeled DDP1 or DDP1C66W precursor was imported into isolated yeast mitochondria for 15 min at 25°C. Mitochondria were either energized (+Δψ) or the mitochondrial membrane potential was dissipated prior to import using valinomycin/FCCP (-Δψ). When indicated, samples were treated with 100 µg/ml proteinase K (PK) mitochondria.

Figure 5

Stability of imported DDP1 and DDP1C66W precursors. Radiolabeled DDP1 or DDP1C66W precursors were imported into isolated mouse liver mitochondria for 10 min at 25°C. Mitochondria were treated with 100 µg/ml trypsin to remove not imported precursor, reisolated by centrifugation and incubated at 37°C in the presence of a ATP-regenerating system containing creatine kinase and creatine phosphate. Aliquots (each corresponding to 50 µg mitochondrial protein) were removed after the indicated time periods and analyzed after SDS-PAGE and blotting onto nitrocellulose membrane. A Fuji PhosphorImaging system was used for quantification. The signal intensity of radiolabeled imported DDP1 at t = 0 min was set to 100%.

Figure 6

Zinc content of DDP1 and DDP1C66W. MBP was fused to DDP1 and DDP1C66W and, as a control, to the α-fragment of β-galactosidase (α) and expressed in E. coli. A, Affinity-purified MBP fusion proteins were subjected to ICP analysis. The signal intensities at the zinc-specific wavelength of 213.857 nm are shown. B, MBP-DDP1 binds Zn2+ in equimolar
amounts. Both MBP-DDP1C66W and MBP-α contained only background levels of Zn2+. Protein concentrations were determined by Bio-Rad protein assay, and molar ratios (mol of Zn2+ per mol of fusion protein) were calculated.

Figure 7

Sensitivity of recombinant DDP1 and DDP1C66W to digestion with trypsin. His-tagged versions of DDP1 and DDP1C66W were expressed in E. coli and affinity-purified on Ni-NTA columns. Purified, recombinant protein (2 µg) was subjected to digestion in 200 µl PBS buffer using increasing amounts of trypsin and analyzed by SDS-PAGE and immunoblotting using a monoclonal antibody against penta-his. When indicated, recombinant protein was incubated with the metal-chelating agents EDTA/o-phe prior to protease treatment.

Figure 8

Oligomeric state of DDP1 and DDP1C66W. A, Gel filtration analysis. Affinity-purified hisDDP1 or hisDDP1C66W (5 µg) were subjected to gel filtration analysis on a Superdex-75 column. Elution fractions were analyzed by SDS-PAGE and immunoblotting using a penta-his antibody and quantified by densitometry. HisDDP1 eluted corresponding to an apparent molecular mass of 65 kDa. HisDDP1C66W was predominantly found in the void volume (not shown). B, Calibration standard curve using cytochrome c, carbonic anhydrase and bovine serum albumin as calibration standards. C, Chemical crosslinking of wild-type and mutant DDP1. Two µg-aliquots of affinity-purified and desalted hisDDP1 or hisDDP1C66W were incubated in the absence or presence of the bifunctional amino-reactive crosslinker DSS at a concentration of 100 µM for 30 min at room temperature. TCA-precipitated crosslinking
products were analyzed by SDS-PAGE and immunoblotting using a penta-his antibody. Arrows indicate cross-linking adducts of hisDDP1 most likely corresponding to dimers, trimers, tetramers, pentamers and hexamers.

**Figure 9**

C66W exchange impairs the interaction between DDP1 and human Tim13. A, hisDDP1 was expressed in *E. coli* and pre-bound to a Ni-NTA resin in order to generate a DDP1-affinity column. The pre-bound resin was then incubated overnight with *E. coli* lysate containing hTim13-GST. After extensive washing, bound material was eluted using 300 mM imidazole and aliquots of elution fractions were analyzed by immunoblotting using affinity purified antibodies against DDP1 and hTim13. hTim13-GST co-eluted together with hisDDP1 (*left panel*).

In a second purification step, the peak elution fraction was incubated with GSH beads. After extensive washing, the entire material bound to GSH beads was analyzed by SDS-PAGE and immunoblotting (*middle panel*) or Coomassie blue staining (*right panel*). The bound material contained hTim13-GST and hisDDP1 in about equimolar amounts (*right panel*). B, *E. coli* lysate containing hTim13-GST was loaded onto a Ni-NTA column to which recombinant hisDDP1C66W was pre-bound. Bound material was eluted and aliquots of elution fractions were analyzed by immunoblotting using anti-DDP1 and anti-hTim13 antibodies. Elutions fraction contained hisDDP1C66W but only trace amounts of hTim13-GST (*left panel*). To exclude that minor amounts of hTim13-GST were bound to hisDDP1C66W, pooled peak elution fractions were applied to GSH beads. Material released from GSH beads was analyzed by immunoblotting (*right panel*).
Fig. 1

A

YPD
15°C

WT
Δ8/Δ13
Δ8/Δ13+
C66W/hTim13
Δ8/Δ13+
DDP1/hTim13

B

\[ A_{578} \text{ (relative cell number)} \]

\[ \text{Time (h)} \]

\[ 24 \quad 48 \quad 72 \quad 98 \quad 120 \]

- ▲ WT
- ○ Δ8/Δ13
- ● Δ8/Δ13+
- ■ Δ8/Δ13+
  - C66W/hTim13
Fig. 2

\[ \Delta 8/\Delta 13^+ \]

DDP1/hTim13

\[ \Delta 8/\Delta 13^+ \]

C66W/hTim13

- PK

- PK

- hTim23
Fig. 7

| Trypsin (µg/ml) | his-DDP1 | his-DDP1 + EDTA/o-Phe | his-DDP1^C66W |
|----------------|---------|----------------------|----------------|
| 0              |         |                      |                |
| 1              |         |                      |                |
| 10             |         |                      |                |
| 50             |         |                      |                |
| 100            |         |                      |                |
| 500            |         |                      |                |
Fig. 9

A

Elution fractions
2 3 4 5 6

anti-hTim13

anti-DDP1

Ni-NTA

GSH beads

Western blotting

hTim13 GST

his-DDP1

GSH beads

Coomassie

B

Elution fractions
2 3 4 5 6

anti-hTim13

anti-DDP1

Ni-NTA

GSH beads

Western blotting

hTim13 GST

his-DDP1<sup>C66W</sup>
The C(66)W mutation in the deafness-dystonia peptide 1 (DDP1) affects the formation of functional DDP1/TIM13 complexes in the mitochondrial intermembrane space

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