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MTERF2 is a nucleoid component in mammalian mitochondria

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ABSTRACT

The mammalian MTERF family of proteins has four members, named MTERF1 to MTERF4, which were identified in homology searches using the mitochondrial transcription termination factor, mTERF (here denoted MTERF1) as query. MTERF1 and MTERF3 are known to participate in the control of mitochondrial DNA transcription, but the function of the other two proteins is not known. We here investigate the structure and function of MTERF2. Protein import experiments using isolated organelles confirm that MTERF2 is a mitochondrial protein. Edman degradation of MTERF2 isolated from stably transfected HeLa cells demonstrates that mature MTERF2 lacks a targeting peptide (amino acids 1–35) present in the precursor form of the protein. MTERF2 is a monomer in isolation and displays a non sequence-specific DNA-binding activity. In vivo quantification experiments demonstrate that MTERF2 is relatively abundant, with one monomer present per ~265 bp of mtDNA. In comparison, the mtDNA packaging factor TFAM is present at a ratio of one molecule per ~10–12 bp of mtDNA. Using formaldehyde cross-linking we demonstrate that MTERF2 is present in nucleoids, and therefore must be located in close proximity to mtDNA. Taken together, our work provides a basic biochemical characterization of MTERF2, paving the way for future functional studies.

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1. Introduction

The mammalian mitochondrial DNA (mtDNA) is a circular double-stranded molecule of ~16 kb, which is organized in nucleoprotein particles called nucleoids [1]. The mitochondrial genome encodes for a small but essential number of genes necessary for the biological function of the organelle. However, all the trans-active factors directly involved in mtDNA replication, transcription, and translation are specified by nuclear genes. Therefore, there must be a regulated communication between the mitochondrial and nuclear genomes [2,3]. Light strand (L-strand) transcription initiates from one single promoter (*L*ight *S*trand *P*romoter or LSP) located in a non-coding region of mtDNA called the control region, while heavy strand (H-strand) transcription is initiated from two different promoters (*H*eavy *S*trand *P*romoter 1 and 2 or HSP_1 and HSP_2). HSP_1 is also located in the control region, whereas HSP_2 is located at the 3′-end of the $tRNA^{Phe}$ coding gene, ~100 bp downstream of HSP_1 [4]. Transcription of the mtDNA from LSP and HSP_2 promoters gives rise to two polycistronic precursor RNA species, which encompass almost the entire length of the mtDNA. The two transcripts are subsequently processed to produce the individual mRNAs, rRNAs and tRNAs. Transcription initiation at HSP_1 starts directly upstream of the $tRNA^{Phe}$ gene and spans the $tRNA^{Phe}$, 12S rRNA, 16S rRNA, and $tRNA^{Val}$ genes [5].

The basic components of the mammalian mitochondrial transcription machinery are the mitochondrial RNA polymerase (POLRMT), the mitochondrial transcription factor A (TFAM), and the mitochondrial transcription factor B1 or B2 (TFB1M or TFB2M). In combination, these factors can initiate transcription from mtDNA promoters in vitro [6]. TFAM is an abundant high-mobility-group-box protein with the ability to bind and bend the mtDNA [7]. In addition to its role as a basal transcription factor [8], TFAM also has a structural role as it contributes to the packaging of mtDNA into nucleoids [9,10]. A specific transcription termination event takes place downstream of the 16S rRNA gene and is mediated by the mitochondrial transcription termination factor MTERF1 (also known as mTERF), which binds a 28 bp region within the $tRNA^{Leu\ (UUR)}$ gene [11,12]. MTERF1 has been shown to terminate transcription in an in vitro transcription system using purified recombinant proteins [13]. In addition, MTERF1 has been reported to interact with a site in close proximity to the HSP_1 promoter [14]. This simultaneous binding of the protein to its two binding sites has been suggested to create a loop that facilitates re-initiation of this transcription unit. Besides its role in mtDNA transcription, MTERF1 has been reported to act as modulator of mtDNA replication in human cells [15].

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Similarity searches have identified three novel genes in vertebrates coding for homologues to MTERF1 (denoted MTERF2, MTERF3, and MTERF4), all of them with predicted mitochondrial localization [16]. MTERF3 is localized to the mitochondrial matrix where it interacts with the mitochondrial promoter region and negatively regulates transcription initiation [17]. Consequently, depletion of the MTERF3 protein in heart mitochondria of knock-out mice leads to a dramatic increase in promoter proximal transcripts. MTERF3 does not appear to terminate mitochondrial transcription, but rather regulates transcription initiation events, although the exact mechanism remains to be elucidated.

MTERF2 is another member of the MTERF family, which colocalizes with mitochondria and has a high expression in heart, liver and skeletal muscle [18]. Transcription of the MTERF2 gene was decreased upon addition of serum in serum-starved cells, which led to suggest that it is a novel serum-inhibitory factor, participating in the regulation of mtDNA transcription. We here present a biochemical characterization of the MTERF2 protein. Our analyses reveal that MTERF2 is a mitochondrial protein, produced as a precursor form and cleaved upon import to the organelle. MTERF2 binds DNA in a non sequence-specific manner and using formaldehyde cross-linking we demonstrate that MTERF2 is present in nucleoids and therefore operates in close proximity to mtDNA.

2. Materials and methods

2.1. Mitochondrial protein import assay

The cDNA encoding the full-length human MTERF2 protein was cloned into pBluescript II SK(+) (Stratagene). The recombinant plasmid was used to express the MTERF2 protein in vitro using the TNT® Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions and in the presence of [^{35}S]-methionine. Mitochondria were freshly isolated from rat livers by differential centrifugation and mitochondrial import assay was performed as described in [17].

2.2. hMTERF2-FLAG protein purification and Edman degradation

The cDNA encoding the full-length human MTERF2 protein with a FLAG-tag at its C-terminus was cloned into pTRE-Tight (Clontech) to obtain the pTRE-FLAG2 vector. HeLa TetOn cells (Clontech) were stably co-transfected with pTRE-FLAG2 and a linear hygromycin resistance marker following the manufacturer's instructions. Cells were grown in monolayer in D-MEM medium supplemented with 10% fetal calf serum, 1% streptomycin, 1% penicillin, and 200 μg/ml hygromycin. The expression of hMTERF2-FLAG was induced by adding 3 μg/ml doxycycline to the incubation medium, and cells were harvested after 72 h of induction. Approximately 15×10^8 HeLa cells were harvested and homogenized using a tight-fitting Dounce homogenizer. The hMTERF2-FLAG protein was purified from mitochondria isolated by differential centrifugation using ANTI-FLAG M2 affinity gel (Sigma) according to the manufacturer's manual. Automated chemical protein sequencing (Edman degradation) was done using a Procise 494 instrument from Applied Biosystems [19] as described [20]. Stepwise-liberated PTH-amino acids were identified using a "on-line" HPLC system [19] equipped with a PTH C18 (2.1×220 mm; 5 mm particle size) column [19].

2.3. hMTERF2-6× His protein purification

A DNA fragment encoding the mature form of the human MTERF2 (that is, the full length protein lacking amino acids 1–35) and with a C-terminal 6× His-tag was cloned into pET-20a(+) (Stratagene) and expressed in *E. coli* strain BL21(DE3) pLysS under standard conditions. Recombinant MTERF2 was first purified under native conditions through a manually packed column containing Ni^{2+}-NTA matrix superflow (Qiagen) and according to the manufacturer's instructions. The peak of eluted protein was dialyzed against Buffer A [25 mM NaH_2PO_4 (pH 7.0), 0.2 M NaCl, 0.5 mM EDTA (pH 8.0), 10% glycerol, 1 mM DTT, proteases inhibitors] and further purified on a 1-ml HiTrap Heparin column (GE Healthcare) equilibrated in Buffer A. We used a linear gradient (10 ml of Buffer A from 0.2 to 1.2 M NaCl) to elute the protein and the fractions in which MTERF2 was eluted (between 0.4 and 0.5 M NaCl) were pooled together.

2.4. Gel filtration chromatography

MTERF2 oligomerization studies were performed by gel filtration chromatography on a Superose 6 column (GE Healthcare) in Gel Filtration Buffer [25 mM Tris–HCl (pH 7.6), 0.2 M NaCl, 3% glycerol, 1 mM DTT, and 0.5 mM EDTA]. 0.5 ml of the eluate from the heparin purification step (described above) was dialyzed for 4 h in Gel Filtration Buffer and then loaded on the Superose 6 column at a flow rate of 0.4 ml/min at 4 °C. Fractions of 0.2 ml were collected, and the protein content was analyzed by SDS-PAGE and Coomassie blue staining. A calibration curve was prepared, following the instructions of the column manufacturer, by running thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa), all from BioRad. The elution of marker proteins was monitored by UV photometry (280 nm). The logarithm of molecular weight was plotted against Kav, which was calculated for each protein as follows: $Kav = (Ve - Vo) / (Vt - Vo)$, where $Ve =$ elution volume for the protein, $Vo =$ column void volume (7.4 ml), and $Vt =$ total bed volume, which is 24 ml for Superose 6.

2.5. Electrophoretic Mobility Shift Assay (EMSA)

The dsDNA binding capacity of the mature MTERF2 protein expressed in bacteria was assayed by EMSA using dsDNA probes covering the entire human mtDNA. The DNA fragments were amplified by PCR using human mtDNA as template, purified and labeled at the 5′ end by using polynucleotide kinase in the presence of [γ-^{32}P]-ATP. A 260 bp fragment in the control region was chosen to further analyze the non-specific DNA interaction. This fragment was amplified using the following primers: Forward 5′-CCACCATCCTCCGTGAAATC-3′; Reverse 5′-CAATGCTATCGCGTGCATAC-3′. Reactions were carried out in 20 μl volumes containing 20 fmol labeled DNA template, 20 mM Tris–HCl (pH 8.0), 0.1 mM DTT, 0.1 mg/ml BSA, 10 mM MgCl2, 5% glycerol, and the protein concentrations indicated in the figure legend. Poly (dI-dC)-poly (dI-dC) was used as dsDNA competitor at a final concentration of 0.1 μg/μl. Reactions were incubated at room temperature for 15 min before separation in non-denaturing polyacrylamide TBE gels of different percentages (6–10%) according to the DNA probe size. The gels were dried used to expose a Phosphorimager screen, which was scanned with a Personal Molecular Imager FX (Bio-Rad). Quantification was carried out with Quantity One 4.6 software (Bio-Rad). The apparent K_d was calculated as described [21]. Identical conditions were employed to study the MTERF1–MTERF2 interactions, but in this case the probe was obtained by annealing the following oligos labeled at their 5′ ends: L-strand 5′-AGA ACA GGG TTT GTT AAG ATG GCA GAG CCC G-3′; H-strand 5′-GG ATT ACC GGG CTC TGC CAT CTT AAC AAA CC-3′.

2.6. Preparation of cross-linked nucleoids from mouse liver mitochondria

Mitochondria were purified from two adult mouse livers (~3 g of tissue) by differential centrifugation. The crude mitochondrial fraction was resuspended in 20 ml MSH Buffer [210 mM Mannitol, 70 mM Sucrose, 20 mM Hepes (pH 8), 2 mM EDTA, proteases inhibitors] and layered onto preformed Percoll/Nycodenz step gradients. The gradients consisted of a first layer of 35% Nycodenz (Axis-Shield), followed

by a second one of 15% Nycodenz and a top layer of 10% Percoll (Sigma), all in MSH Buffer. After the gradients were centrifuged at 25,000 rpm for 25 min in a Beckman SW32 rotor, the mitochondrial fraction was collected from the 15/35% Nycodenz interface, diluted four times with MSH Buffer, 0.1 ml/ml BSA was added, and finally pelleted by centrifugation at 20,000 ×*g* for 15 min. The crude mitochondrial pellet was first treated with nucleases and then highly purified through a sucrose gradient as described in [22]. Pure mitochondria were resuspended in 2 ml MSH Buffer and pelleted twice (20,000 ×*g* for 5 min) to wash away the sucrose residues. Following the last centrifugation, formaldehyde cross-linking and preparation of the mtDNA nucleoids were performed as described in [23]. Fractions of ~1 ml were collected from the top to the bottom of the glycerol gradient, and assayed for DNA and protein content. More precisely, 0.3 ml of each gradient fraction was mixed with 0.3 ml of TES Buffer [10 mM Tris–HCl (pH 8), 1 mM EDTA, 1.5% SDS] and incubated overnight at 65 °C to reverse the formaldehyde cross-links. About 0.4 ml of each reversed cross-link fractions was assayed for mtDNA content; DNA was purified by extraction with phenol–$CHCl_3$, precipitated with ethanol, and linearized with Sac I restriction endonuclease. mtDNA was visualized by ethidium bromide staining after separation on agarose gel. The remaining amount of the reversed cross-link fractions was analyzed for protein content. Proteins were precipitated with 25% trichloroacetic acid (TCA) and 0.5 mg/ml of Na-deoxycholate (DOC) on ice for 20 min. After 20 min centrifugation at 20,000 ×g, 4 °C, the protein pellets were washed with 200 µl of ice-cold acetone, centrifuged as above for 5 min, air-dried and redissolved in 25 µl of 1× Laemmli Buffer. Proteins were separated in a 4–20% gradient SDS-PAGE gel and Western blot analysis was performed as described later.

2.7. Western blot analysis

Livers were collected from adult mice and crude mitochondria were prepared by differential centrifugation. Mitochondrial protein content was determined by Bradford assay. Mouse liver mitochondria were resuspended in 2× Laemmli Buffer for 5 min at 95 °C and separated on a 12.5% SDS-PAGE gel. Western blot analysis was performed as described [9] to determine the protein levels of mouse MTERF2 and TFAM. The full-length recombinant mouse MTERF2 protein was expressed and purified from bacteria and used to immunize rabbits. The antisera used in this study were taken after the second booster injection (Agrisera AB, Sweden). The primary antibodies were diluted 1:500 for anti-mMTERF2 and 1:1000 for anti-mTFAM. The quantification of mMTERF2 and mTFAM was performed by interpolating the intensity of their bands in the Western blot in a calibration curve made with known amounts of recombinant proteins using the Quantity One 4.6 software (Bio-Rad).

3. Results

3.1. MTERF2 is imported into mitochondria and cleaved upon import

We first studied the uptake of recombinant [^{35}S]-methionine-labeled human MTERF2 into isolated rat mitochondria (Fig. 1A). Import of full-length MTERF2 into mitochondria generated a protein species of lower molecular weight, indicating that the protein contains a mitochondrial targeting sequence that is cleaved after import. This process was inhibited by the addition of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), consistent with the fact that mitochondrial protein import across the inner membrane is dependent on the mitochondrial membrane potential. Additionally, the processed MTERF2 protein was resistant to trypsin treatment, while the full-length precursor form was degraded. These data show that MTERF2 is synthesized in the cytosol and contains an N-terminal peptide that directs the protein towards the mitochondria. The mitochondrial targeting sequence is cleaved after import to give rise to the mature mitochondrial form of the protein.

3.2. Identification of the N-terminus of the mature MTERF2 protein

To identify the N-terminal sequence of the mature protein and to investigate the existence of MTERF2 interacting proteins, we established stable HeLa cell lines expressing FLAG-tagged human MTERF2 protein. MTERF2-Flag expression was induced by the addition of doxycycline and the protein expression showed a linear correlation with the amount of added inducer (Fig. 1B). FLAG-tag affinity purification was used to isolate the protein from total HeLa cell lysate. Two major protein species were eluted (Fig. 1C) and MALDI-TOF analysis identified one of them as the human MTERF2, while the other

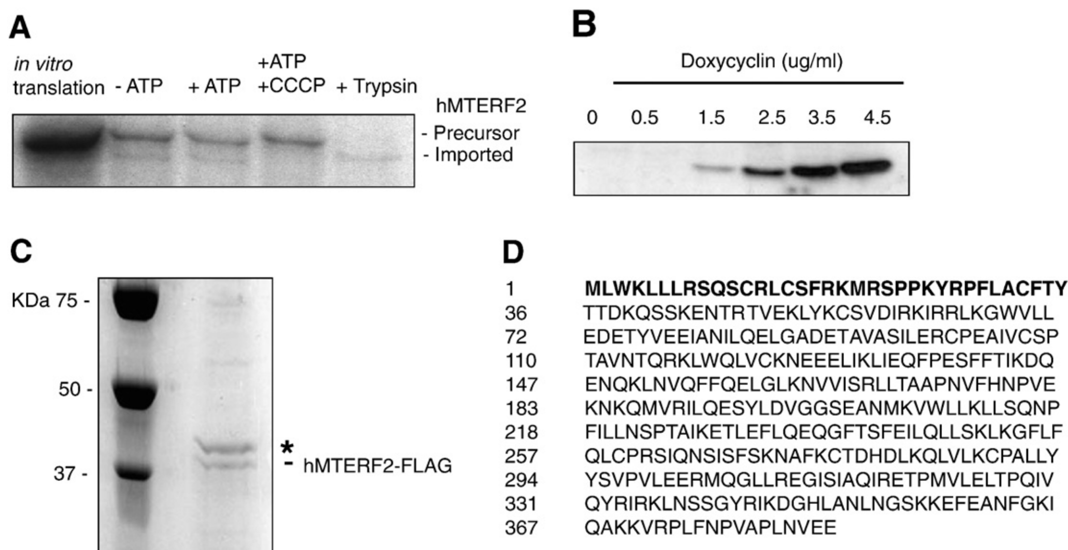

Fig. 1. MTERF2 mitochondrial localization and identification of its mitochondrial targeting sequence. (A) Import of radiolabeled human MTERF2 protein (hMTERF2) into isolated rat mitochondria. (B) HeLa cells, stably transfected with a plasmid encoding the FLAG-tagged human MTERF2 protein (hMTERF2-FLAG), show doxycyclin-dependent overexpression of the MTERF2 protein. The MTERF protein was identified by immunoblotting using a polyclonal antibody. (C) Affinity purification of the hMTERF2-FLAG protein. The asterisk indicates the human β-actin. (D) A 35 amino acid long peptide is cleaved from the full-length human MTERF2 protein, upon import into mitochondria. The mitochondrial targeting sequence is shown in bold.

one was human β-actin. As one of the main components of the cytoskeleton, β-actin plays an active role in organelles movements within eukaryotic cells. However, since β-actin has not been shown to be present within mitochondria, the functional relevance of the observed interaction is questionable and therefore it is most likely an artifact of the purification procedure.

We used Edman degradation to identify the N-terminus of the mature MTERF2 protein and found that the processing site was localized between amino acid positions 35 and 36 of human MTERF2 (Fig. 1D). The mature human MTERF2 protein is thus 350 amino acid long after removal of the N-terminal peptide, which contains a number of hydrophobic and positively charged residues typical for eukaryotic mitochondrial targeting sequences [19].

3.3. MTERF2 binds mtDNA

We expressed a His-tagged version of the mature form of the protein in *E. coli* to obtain recombinant protein for characterization of the biochemical properties of MTERF2. The recombinant protein was purified to near homogeneity, as estimated by Coomassie blue staining (Fig. 2A). Gel filtration chromatography analysis was employed as a last step in the purification procedure (Fig. 2B) and was also used to study the oligomerization status of MTERF2. In the latter case, MTERF2 was run in parallel with standard proteins of known molecular weight (Fig. 2C). Our analysis showed that MTERF2 has an apparent molecular mass of ~44 kDa, which closely corresponds to the calculated theoretical molecular weight of 40.1 kDa. We thus conclude that MTERF2 most likely is a monomer in solution.

Next, we employed Electrophoretic Mobility Shift Assays (EMSA) to investigate the DNA-binding activity of recombinant MTERF2. The protein displayed a strong non-sequence-specific DNA-binding activity (Fig. 3A), with an apparent K_d of about 18 nM (Fig. 3B). Competitor dsDNA [poly (dI-dC)-poly (dI-dC)] could efficiently disrupt the observed MTERF2-DNA complex. We used two different approaches to identify mtDNA sequences, which could be specifically bound by MTERF2. First, we performed EMSA assays using 32 DNA fragments covering the entire human mtDNA. Second, we performed genome-wide chromatin immunoprecipitation (ChIP) assays with 31 primers pairs covering the entire mouse mitochondrial genome, as previously described for MTERF3 [17]. Neither of these two approaches showed any sequence-specific binding of MTERF2 to mtDNA (data not shown).

Since MTERF2 is structurally related to MTERF1 [16,18], we speculated that MTERF2 could interact with and influence the DNA binding activity of the MTERF1 protein. Human MTERF1 binds mtDNA as a monomer [11] and to test if MTERF2 could interact with MTERF1, we performed EMSA on a DNA template containing the MTERF1 binding site. MTERF1 could shift the labeled probe in the presence of a non-specific competitor, but addition of MTERF2 did not cause a further shift of the MTERF1-DNA complex (Fig 4). We therefore concluded that MTERF2 does not interact with MTERF1 bound to DNA. This result was also in agreement with our observation that MTERF1 does not interact with FLAG-MTERF2 purified from HeLa cells (Fig 1C).

3.4. MTERF2 is an mtDNA nucleoid protein

We next explored the possibility that MTERF2 could associate with mtDNA nucleoids. To this end, mouse liver mtDNA nucleoids were

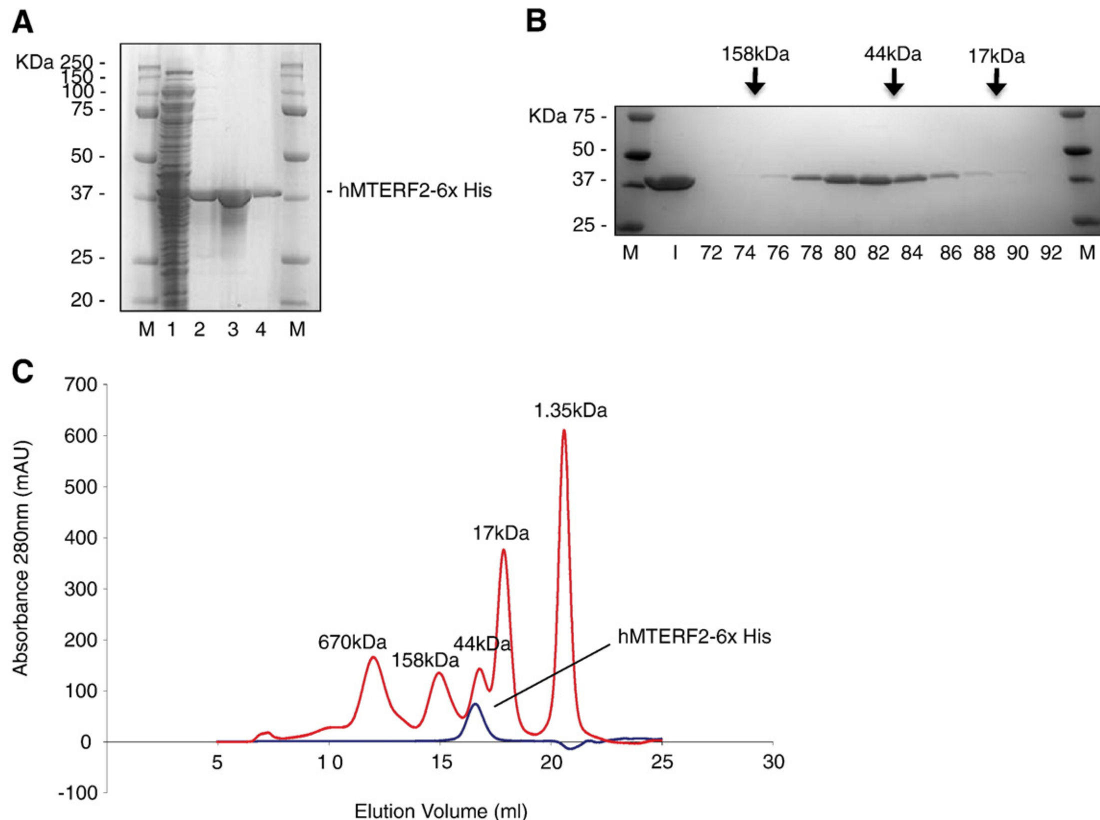

Fig. 2. Purification of the recombinant human MTERF2 expressed in bacteria. (A) The human MTERF2 protein lacking amino acids 1–35 and with a 6×-His-tag at its C-terminus (hMTERF2-6× His), was over-expressed in *E. coli* and purified to homogeneity. M: molecular size marker (Precision Plus Protein Standard — Dual Color, Bio-Rad); lane 1: bacterial crude extract after induction of hMTERF2-6× His expression; lane 2: Ni^{2+}-affinity purification; lane 3: heparin purification; lane 4: gel filtration purification. (B) The hMTERF2-6× His protein was analyzed on a Superose 6 gel filtration column equilibrated with 0.2 M NaCl and the fractions around the elution peak were visualized on SDS-PAGE, stained with Coomassie blue. M: molecular size marker; I: input. Fraction numbers are indicated below the figure, while the approximate elution fraction of some of the protein standards is indicated on the top. (C) Elution curves of the protein standards (red) and hMTERF2-6× His protein (blue) after gel filtration.

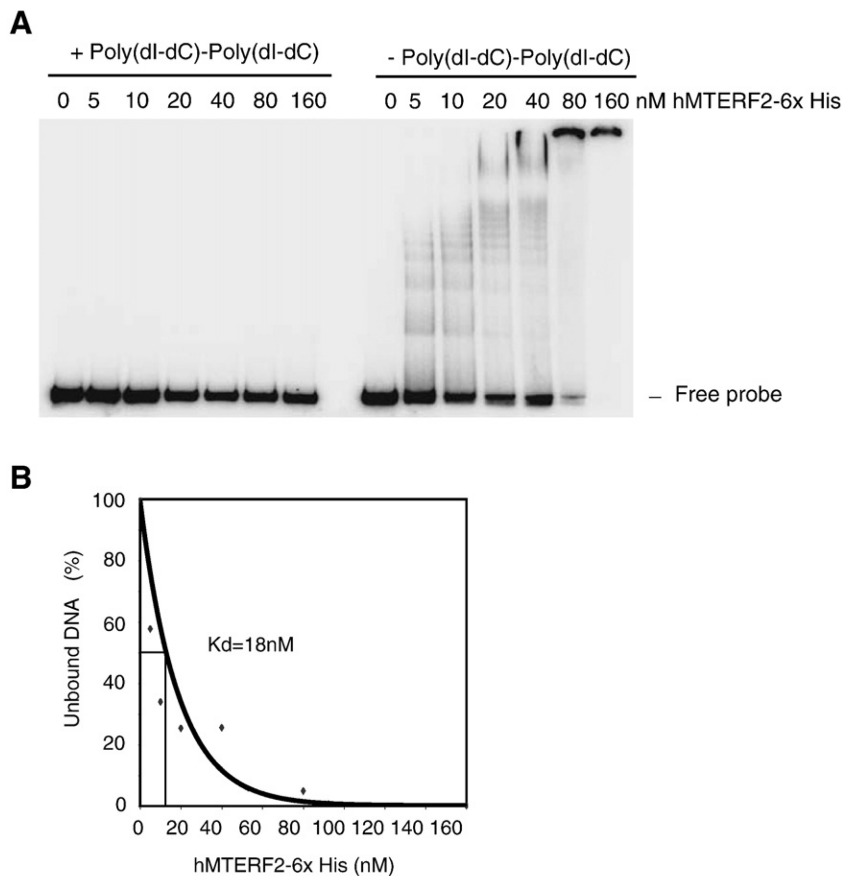

Fig. 3. MTERF2 binds mtDNA. (A) Electrophoretic Mobility Shift Assays (EMSA) revealed a non sequence-specific DNA-binding activity of the hMTERF2-6× His protein. The probe used in this experiment is a 260 bp fragment of the human mitochondrial control region (16400–91 according to GenBank J01415), but similar results were obtained with 32 different probes covering the entire human mtDNA (data not shown). (B) Measurement of the apparent K_d for the interaction between MTERF2 and DNA.

prepared using a modification of the formaldehyde cross-linking protocol, as described [23]. Mitochondrial purification was carried out as described in Materials and methods and 1% formaldehyde was used to cross-link proteins associated with mtDNA. After mitochondrial lysis, the mtDNA-proteins complexes were separated from free proteins by sedimentation through a glycerol/Nycodenz gradient. Following sedimentation, mtDNA was found in a peak in the centre of the gradient (Fig. 5A) and was associated with TFAM (Fig. 5B). TFAM molecules not cross-linked to mtDNA remained at the top of the gradient together with the vast majority of mitochondrial proteins. When the same gradient fractions were assayed for MTERF2, a similar pattern to the one shown for the TFAM protein was observed (Fig. 5B). We conclude that MTERF2 is found in association with purified mtDNA nucleoids and co-localizes with TFAM. MTERF2 is thus a novel component of mtDNA nucleoids.

3.5. MTERF2 is an abundant protein

In order to quantify the levels of MTERF2 in vivo, we isolated mitochondria from mouse liver and performed quantitative Western

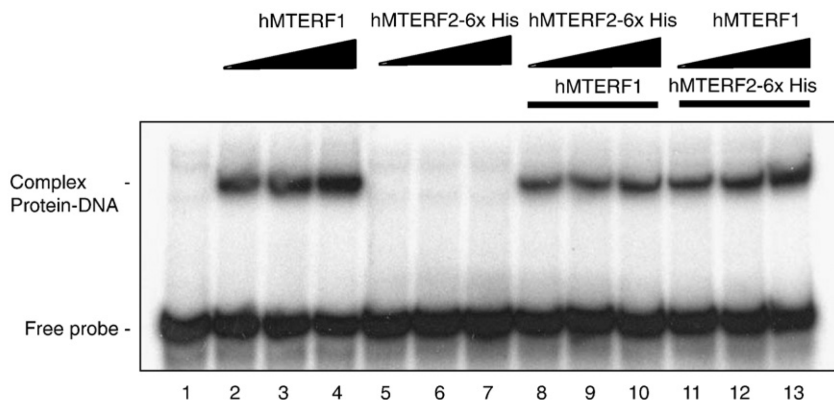

Fig. 4. MTERF1–MTERF2 interaction. EMSA was employed to test MTERF1–MTERF2 interactions. Recombinant human MTERF1 (hMTERF1) interacted with its cognate binding site (lines 2 to 4). The hMTERF2-6× His does not bind the probe either alone (lines 4 to 7) or in combination with hMTERF1 (lines 8 to 13). Lane 1: reaction mixture containing 20 fmol of labeled DNA; lanes 2–4: 0.5, 1 and 2 pmol of hMTERF1 were added to the reaction mixture; lanes 5–7: 0.5, 1 and 2 pmol of hMTERF2-6× His were used in the reaction mixture; lanes 8–10: increasing amounts of hMTERF2-6× His (0.5, 1 and 2 pmol) were used in combination with constant amount of hMTERF1 (0.5 pmol); increasing amounts of hMTERF1 (0.5, 1 and 2 pmol) were used in combination with constant amount of hMTERF2-6× His (0.5 pmol).

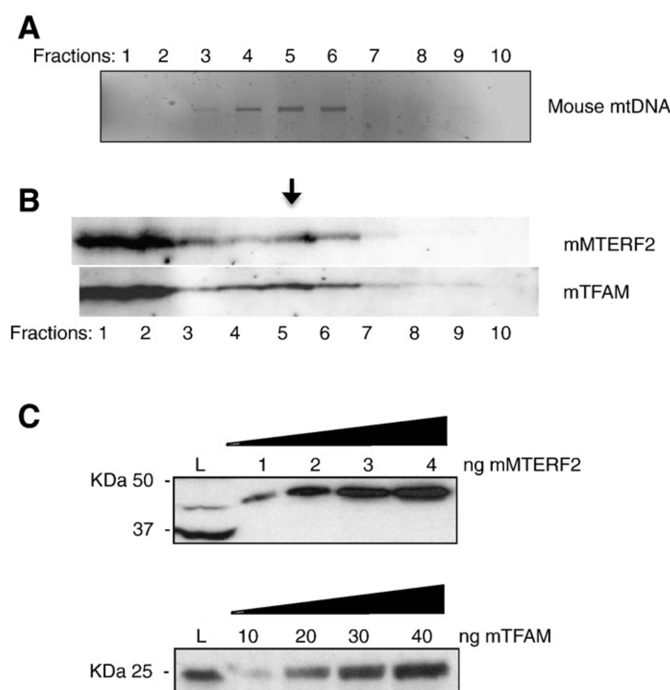

Fig. 5. MTERF2 is an abundant mitochondrial protein localized in the mtDNA nucleoids. (A) Glycerol gradient sedimentation of cross-linked mouse liver mitochondria. Fractions were collected from the top to the bottom of the tube and the formaldehyde cross-links were reversed by heat treatment. (A) mtDNA was linearized with Sac I and visualized by EtBr. (B) Fractions containing mouse MTERF2 (mMTERF2) or mouse TFAM (mTFAM) were visualized by Western blotting. (C) The endogenous levels of mMTERF2 and mTFAM in 30 μg of mouse liver mitochondrial lysate (L) were determined by Western blot using as standards the indicated amounts of the full-length mouse recombinant MTERF2 (mMTERF2) and the mouse recombinant TFAM in its mature form (mTFAM).

blot analysis using polyclonal antisera against the mouse MTERF2 protein (Fig. 5C). The level of mouse MTERF2 protein was determined by comparison with known amounts of recombinant protein. The levels of MTERF2 corresponded to ~0.07–0.1 ng per μg of total mitochondrial protein. The relative levels of mouse TFAM protein were determined in the same way and found to be ~1–1.5 ng per μg of total mitochondrial proteins. We also performed additional experiments to calculate the ratio of MTERF2 protein to mtDNA in isolated mouse liver mitochondria. We used a combination of Western and Southern blot analysis together with recombinant mouse MTERF2 protein and plasmid DNA standards (data not shown). Our data revealed a ratio of one molecule of MTERF2 per ~265 bp of mtDNA. Using the same approach we could measure a ratio of one molecule of mouse TFAM per ~10–12 bp of mtDNA, which is in very good agreement with previous estimates in mouse heart mitochondria [9].

The relatively high abundance of MTERF2, together with the observation that it binds mtDNA, suggested that the protein could regulate processes such as mtDNA replication or transcription. To address these possibilities, we analyzed the effects of MTERF2 on DNA replication using a previously described in vitro mtDNA replication system, where a mini-circle template and recombinant human mitochondrial replication factors were employed [24]. Addition of the recombinant MTERF2 protein expressed in bacteria did not affect the mitochondrial DNA replication in vitro (data not shown). Similarly, addition of the MTERF2 protein failed to affect in vitro transcription from the LSP or HSP promoters using previously described conditions [6]. MTERF2 does not directly affect mitochondrial replication or transcription in the tested in vitro experimental conditions. Of course, these findings do not exclude that MTERF2 has a role in mtDNA replication or transcription in vivo.

4. Discussion

MTERF1 has the capacity to terminate transcription initiated from HSP_2 in a recombinant in vitro system, but there is as yet no in vivo experimental support for this mechanism. The A3243G mutation is a common cause of mitochondrial encephalopathy, lactic acidosis as stroke-like episodes (MELAS) syndrome and has been reported to decrease the affinity of MTERF1 for its DNA-binding site significantly. However, patients harboring the A3243G mutation show no change in levels of upstream or downstream mature mtDNA transcripts [25] and in vivo footprinting could not reveal any significant difference in MTERF1 occupancy between mutant and wild-type mtDNAs [26].

The exact mechanisms of HSP_1 dependent transcription termination therefore remain obscure and other factors may influence HSP_1-dependent transcription termination in vivo. In addition, LSP- and HSP_2-dependent transcription must also be terminated and these may also be regulated events. Given the homology between MTERF1 and the other members of the MTERF family, the other MTERFs are interesting candidates for studies of transcription termination in mammalian mitochondria. The striking sequence similarity between MTERF1 and MTERF2 evokes the idea that the two proteins can contribute to transcription termination at the MTERF1 binding site. However, the experimental evidence presented here suggests that this is not the case. The MTERF2 protein does not interact with the MTERF1 binding site and does not influence MTERF1 dependent transcription termination in vitro. Future mouse knockout studies should be performed to clarify if MTERF1 and MTERF2 alone or in combination has roles in transcription termination.

The MTERF proteins may also influence other cellular activities. The MTERF family of proteins has recently been linked to the regulation of mtDNA replication [15,27]. The region of the canonical MTERF1-binding site functions as a replication pause site and the strength of pausing is modulated by the expression level of MTERF1 [15]. It is even possible that MTERF2, similar to MTERF3, may have a molecular role not involving termination of transcription or replication of mtDNA. MTERF3 is repressor of transcription initiation [17] and the protein lacks a sequence-specific DNA-binding activity as assessed by EMSA experiments. However, ChIP experiments revealed that the MTERF3 protein interacts with the promoter region of mtDNA. The lack of sequence-specific binding in vitro, could indicate that MTERF3 activity is regulated in some manner, e.g. via posttranslational modifications. Alternatively, MTERF3 binding to the promoter region may be assisted by other factors, e.g. via direct interactions with sequence-specific DNA binding factors.

As reported here, MTERF2 has the capacity to bind to DNA non-sequence-specifically, and it is found in formaldehyde-crosslinked nucleoids. The apparent lack of a sequence-specific DNA binding activity and the physical proximity to mtDNA could suggest a role for MTERF2 in mtDNA packaging. Further work is obviously required in order to directly address this and other hypotheses. We hope that disruption of the MTERF2 gene in mice will shed more light on the molecular function of this conserved protein.

Previous reports did not identify MTERF2 as a nucleoid component [23,28,29]. As demonstrated here, MTERF2 is present at lower abundance than TFAM, which is a hallmark protein of mitochondrial nucleoids. The relative scarcity of the protein may therefore have made identification difficult. In addition, different proteins are more or less easy to detect by mass spectrometry mass fingerprinting, which may leave some important nucleoid components unnoticed.

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