Mitochondrion 15 (2014) 1–9

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Mitochondrial membrane assembly of TMEM70 protein

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ARTICLE INFO

Article history:
Received 21 October 2013
Received in revised form 4 February 2014
Accepted 18 February 2014
Available online 25 February 2014

Keywords:
Mitochondria
ATP synthase
TMEM70
Biogenesis

ABSTRACT

Dysfunction of TMEM70 disrupts the biogenesis of ATP synthase and represents the frequent cause of autosomal recessive encephalocardiomyopathy. We used tagged forms of TMEM70 and demonstrated that it has a hairpin structure with the N- and C-termini oriented towards the mitochondrial matrix. On BN-PAGE TMEM70 was detected in multiple forms including dimers and displayed partial overlap with assembled ATP synthase. Immunoprecipitation studies confirmed mutual interactions between TMEM70 molecules but, together with immunogold electron microscopy, not direct interaction with ATP synthase subunits. This indicates that the biological function of TMEM70 in the ATP synthase biogenesis may be mediated through interaction with other protein(s).

1. Introduction

Mitochondrial ATP synthase is a multisubunit protein formed by F_1 catalytic part and membrane embedded F_o part, that are connected via two stalks. The biosynthesis and assembly of ATP synthase is a complex, stepwise process requiring several specific helper proteins (Ackerman and Tzagoloff, 2005). Although the structure of ATP synthase is highly similar between lower and higher eukaryotes, the biogenesis of the enzyme is rather different reflecting differences in number, expression and processing of mtDNA encoded subunits. Thus 12 specific factors involved in the biosynthesis and assembly of the mtDNA encoded subunits ATP6, ATP8 and ATP9 of the F_o part of the enzyme have been described in *Saccharomyces cerevisiae* (Ackerman and Tzagoloff, 2005; Osman et al., 2007; Zeng et al., 2007a, 2007b, 2008) and none of them has functional homologs in higher eukaryotes. In addition, 3 factors are involved in the assembly of yeast F_1 catalytic part of ATP synthase and two of them have their functional homologs in mammals — Atp11p and Atp12p chaperones for F_1-β and F_1-α subunits respectively (Wang et al., 2001). Recently TMEM70 protein has been described as another ancillary factor in the ATP synthase biogenesis (Cizkova et al., 2008). In contrast to the above-mentioned factors, TMEM70 is only present in higher eukaryotes and is lacking in *S. cerevisiae* and many other lower eukaryotes (Cizkova et al., 2008; Jonckheere et al., 2011).

The functional role of TMEM70 protein was discovered while searching for the disease causing gene responsible for the fatal mitochondrial disease caused by isolated deficiency of ATP synthase (Cizkova et al., 2008; Houstek et al., 1999). Mutation c.317-2A>G in *TMEM70* gene preventing the synthesis of the protein was found to be the cause of ATP synthase deficiency in the group of patients with severe neonatal encephalocardiomyopathy. Since then, several other *TMEM70* mutations have been described (c.118_119insGT, c.494G>A, c.336T>A, c.316+1G>T, c.238C>T, c.578_579delCA, c.211-450_317-568del, c.580G>A, g.2436–3789, c.628A>C and c.535C>T (Atay et al., 2013; Cameron et al., 2011; Cizkova et al., 2008; Jonckheere et al., 2011; Shchelochkov et al., 2010; Spiegel et al., 2011; Torraco et al., 2012; Tort et al., 2011)), demonstrating that *TMEM70* gene is rather prone to mutations and represents the most frequent cause of ATP synthase deficiency.

We have previously described that the 30 kDa precursor of TMEM70 is processed upon import to mitochondria to 21 kDa mature protein with two putative transmembrane domains, and it behaves as an inner mitochondrial membrane protein capable to form dimers or aggregates with some other protein(s) (Hejzlarova et al., 2011). While our previous studies pointed to the involvement of TMEM70 in the early stages of ATP synthase biogenesis, possibly the F_1 formation (Houstek et al., 1999, 2009), Torraco et al. (2012) recently suggested

Abbreviations: AA, amino acids; DDM, n-dodecyl-β-D-maltoside; F_1, catalytic part of ATP synthase; F_o, membrane embedded part of ATP synthase; TX-100, Triton X-100; PIC, protease inhibitor cocktail.

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http://dx.doi.org/10.1016/j.mito.2014.02.010
1567-7249/

the association of TMEM70 protein with ATP synthase subcomplex and the role of TMEM70 in the last steps of ATP synthase assembly — incorporation of the mtDNA encoded subunits F_o-a and A6L. However, the proper conformation of TMEM70 in the membrane as well as its function remain unknown.

In the present study we aimed to characterize structural properties of TMEM70 protein, namely its orientation in the inner mitochondrial membrane, presence in protein complexes under native conditions and its possible direct interactions with ATP synthase or other mitochondrial proteins.

2. Materials and methods

2.1. Cell cultures

Human embryonic kidney cells (HEK293, CRL-1573, ATCC) were grown in high glucose DMEM medium (PAA) supplemented with 10% (v/v) fetal bovine serum (Gold, PAA) at 37 °C in 5% CO_2 atmosphere.

2.2. Expression vectors and transfection

TMEM70-FLAG cDNA was inserted into vector pIRESpuro3 (Clontech) (Hejzlarova et al., 2011) and transfected into the HEK293 cell lines by nucleofection Kit V (Amaxa/Lonza). *TMEM70-GFP* cDNA expression vector (Calvo et al., 2006) was kindly provided by Prof. V.K. Mootha. To generate *TMEM70-MYC-FLAG*, *TMEM70* ORF was PCR amplified from *TMEM70-GFP* cDNA and cloned into the pCMV6-Entry plasmid (Origene). Plasmids were transfected into the HEK293 cell lines using METAFECTENE PRO (Biontex). *TMEM70-GFP* cDNA expression vector was transiently transfected into the HEK293 cells expressing TMEM70-FLAG by Express-In Transfection Reagent (Open Biosystems).

2.3. Isolation of mitochondria

Mitochondria were isolated from freshly harvested cells by hypo-osmotic shock method (Bentlage et al., 1996). Alternatively, for trypsin treatment experiment with TMEM70-FLAG, mitochondria isolated from cells homogenized by a Dounce homogenizer in isotonic medium (Stiburek et al., 2005) were used.

2.4. Trypsinization of the endogenous or C-terminal tagged TMEM70 protein in cells and mitochondria

Freshly harvested HEK293 cells constitutively expressing TMEM70-GFP, TMEM70-MYC-FLAG and control HEK293 cells were suspended in SEKT (0.25 M sucrose, 2 mM EGTA, 40 mM KCl and 20 mM Tris, pH 7.4) to a final protein concentration 5 mg/mL, mixed with the same volume of digitonin (1 mg/mL in SEKT) and incubated for 15 min on ice. The permeabilized cells were then incubated with trypsin (1 or 4 μg/100 μg of protein) in the presence or absence of 1% (v/v) Triton X-100 (TX-100) for 20 min at 37 °C. To stop digestion, SDS sample buffer (2% (v/v) 2-mercaptoethanol, 4% (w/v) SDS, 50 mM Tris (pH 7.0), 10% (v/v) glycerol) was added and the samples were incubated for 5 min at 90 °C. Samples were analyzed by 12% SDS-PAGE and WB using antibodies against TMEM70, FLAG, GFP, and compartment specific markers (intermembrane space — OPA1, matrix — PDH, loading control — porin). For details about antibodies used see Section 2.8.

To analyze the effect of trypsin on isolated organelle, mitochondria isolated from HEK293 cells constitutively expressing TMEM70-FLAG were exposed to hypotonic shock (Tang et al., 2009). Briefly, 150 μg of mitochondrial protein were resuspended in 100 μL of 5 mM MOPS-KOH, pH 7.2, and incubated for 20 min at 4 °C on a rotator. Swollen mitochondria with disrupted outer membrane were treated with trypsin (4 μg/100 μg of protein) for 20 min at 4 °C on a rotator in the presence or absence of 1% TX-100. Digestion was stopped with 100 μg/mL of soybean trypsin inhibitor and 1% (v/v) protease inhibitor cocktail (PIC, Sigma P8340). Samples were analyzed by 12% SDS-PAGE and WB using antibodies against FLAG and compartment specific markers (intermembrane space — OPA1, matrix — fumarate hydratase, inner membrane — Ndufb6).

2.5. Fluorescence microscopy analysis of TMEM70-GFP localization in intact cells and cells with permeabilized cytoplasmic and mitochondrial membrane

Selective permeabilization of the cytoplasmic and mitochondrial membranes by detergents as well as quenching of GFP fluorescence by Trypan blue was utilized as a tool to assess GFP tag orientation in mitochondria, essentially as in De Stefani et al. (2011). We used HEK293 cell lines transiently expressing TMEM70-GFP fusion protein, cells expressing GFP fusion protein localized in the cytosol (pMax-GFP, Amaxa/Lonza), or GFP fusion protein localized in the mitochondrial matrix (mt-GFP, Stratagene). Experiments were performed 48 h after transfection. The fluorescence signal was monitored on Leica DMI6000 wide field microscope with motorized z-stage equipped with Andor iXon897 photon counting camera. For each time point, image z-stack was collected (step optimized to 1 airy) and best focused plane was used for quantification. Cells were imaged in intracellular buffer containing 120 mM KCl, 3 mM HEPES, 5 mM KH_2PO_4, 3 mM $MgSO_4$ and 1 mM EGTA, pH 7.2. Each acquisition lasted for 10 min and subsequent additions were as follows: digitonin (0.2 g/g of protein, at 120 s), proteinase K (4 units, at 240 s), Trypan blue dye (0.05% (w/v), at 360 s) and TX-100 (1%, at 480 s). Total signal intensity from the whole cell at each timepoint was quantified in ImageJ software (FIJI package).

2.6. Immunoprecipitation

For TMEM70-FLAG immunoprecipitation, 1.3 mg protein of freshly isolated mitochondria from cultured cells were lysed with 0.35 mL buffer containing 0.5% TX-100, 150 mM NaCl and 50 mM Tris–HCl (pH 7.4) and 1% PIC for 30 min at 4 °C on a rotator and centrifuged at 12,000 *g* for 10 min. The lysate (0.32 mL) was incubated overnight at 4 °C with previously washed 40 μL of an EZ View ANTI-FLAG M2 affinity agarose resin (Sigma, F2426). Subsequently the resin was washed three times with buffer containing 150 mM NaCl and 50 mM Tris–HCl (pH 7.4). The bound protein was eluted by competition with 3× FLAG peptide (Sigma, F4799). The eluted immunoprecipitate was combined with SDS sample buffer and analyzed by 12% SDS-PAGE and Western blotting.

For ATP synthase immunoprecipitates, ATP Synthase Immunocapture Kit (Abcam, ab109715) was used. Freshly isolated mitochondria (0.45 mg protein) were solubilized with 2 g of n-dodecyl-β-D-maltoside (DDM)/g of protein in 90 μL PBS containing 0.2% PIC for 15 min on ice and centrifuged at 30,000 *g* for 20 min. Supernatant (80 μL) was mixed with ATP Synthase Immunocapture matrix. The mixture was incubated overnight at 4 °C on rotator and subsequently washed three times with PBS + PIC. Washed beads were mixed with SDS sample buffer and the sample subjected to 10% SDS-PAGE and Western blotting.

2.7. Polyacrylamide gel electrophoresis and Western blot immunodetection

SDS-PAGE (Schagger and von Jagow, 1987) was performed on 10% or 12% polyacrylamide minigels using Mini-Protean system (Bio-Rad).

BN-PAGE was performed on 8–15% polyacrylamide minigels (Schagger and von Jagow, 1991). Isolated mitochondria were solubilized with DDM (1 g/g of protein) or digitonin (2 g/g of protein) for 15 min on ice in 1.5 M aminohexanoic acid, 2 mM EDTA and 50 mM Bis-Tris, pH 7.0. The samples were centrifuged for 20 min at 4 °C and 30,000 *g* and Coomassie Brilliant Blue G-250 dye (Serva, 0.1 g/g of detergent) and 5% (v/v) glycerol were added to supernatants before electrophoresis.