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The mitochondrial TMEM177 associates with COX20 during COX2 biogenesis

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ABSTRACT

The three mitochondrial-encoded proteins, COX1, COX2, and COX3, form the core of the cytochrome *c* oxidase. Upon synthesis, COX2 engages with COX20 in the inner mitochondrial membrane, a scaffold protein that recruits metallochaperones for copper delivery to the Cu_A-Site of COX2. Here we identified the human protein, TMEM177 as a constituent of the COX20 interaction network. Loss or increase in the amount of TMEM177 affects COX20 abundance leading to reduced or increased COX20 levels respectively. TMEM177 associates with newly synthesized COX2 and SCO2 in a COX20-dependent manner. Our data shows that by unbalancing the amount of TMEM177, newly synthesized COX2 accumulates in a COX20-associated state. We conclude that TMEM177 promotes assembly of COX2 at the level of Cu_A-site formation.

1. Introduction

Mitochondria produce the majority of cellular energy in form of ATP by oxidative phosphorylation. Therefore, a proton gradient is generated by the respiratory chain. The required energy to build this proton imbalance is provided by oxidation of NADH and $FADH_2$ in the mitochondrial matrix and electron transport through the respiratory chain. Concomitantly, the F_1F_o ATP synthase utilizes the proton gradient to drive ATP synthesis.

The terminal complex of the respiratory chain, cytochrome *c* oxidase, reduces molecular oxygen to water. This enzyme complex is formed by 14 structural subunits in human [1–4]. Eleven subunits are encoded in the nucleus and need to be imported into mitochondria. Three highly conserved core subunits (COX1, COX2 and COX3) are encoded by the mitochondrial genome (mtDNA). These core subunits are translated within mitochondria and co-translationally inserted into the inner mitochondrial membrane [5–9]. COX1 forms the stepping stone of cytochrome *c* oxidase assembly to which imported and mitochondrial-encoded subunits associate in a sequential manner. The current concept suggests that COX2 and COX3 associate with a specific set of assembly factors first and subsequently associate to the COX1-containing module [2,10–12]. Although cytochrome *c* oxidase maturation is considered as a stepwise process through a series of assembly intermediates, assembly stages of COX2 and COX3 are still ill defined.

Our understanding of the COX2 biogenesis process and the involvement of assembly factors has been mainly obtained from studies in the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*). In contrast to human COX2, the yeast homolog is synthesized as a precursor protein [13,14] and its N-terminal leader sequence processed by the inner membrane peptidase (IMP) complex [15–17]. Mature Cox2 contains two transmembrane domains and exposes its N- and C-terminus to the intermembrane space. Several Cox2-specific assembly factors have been described in yeast including Pet111, Cox18 and Cox20. While Pet111 acts as Cox2 translational activator [18–20] and promotes co-translational membrane insertion of the Cox2 N-terminus, Cox18 [21–24] and Cox20 [14,25] are involved in C-terminal translocation. In addition, the interaction of Cox20 with Cox2 is required to promote Cox2 association with the metallochaperone Sco1 [26–28], essential for Cu_A-side maturation.

In *S. cerevisiae* Cox20 is present in distinct complexes with different protein compositions [29]. All Cox20-complexes contain Cox2. However, distinct complexes of Cox20 appear to engage with ribosomes and the copper chaperone Sco1. These findings imply that Cox20 fulfills

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several functions during Cox2 assembly.

SCO1 and SCO2, both human homologs of the yeast Sco1, have been described as COX20-associated proteins and are essential factors for cytochrome *c* oxidase maturation [30–33]. Similar to COX20 [34,35], also SCO1 and SCO2 have been associated with mitochondrial disorders, such as muscle hypotonia, ataxia, encephalocardiomyopathies, hepatopathies and defined cytochrome *c* oxidase deficiency [36–40].

COX20 has been found to form a complex with SCO1, SCO2, and newly synthetized COX2 [41]. However, a comprehensive analysis of the interaction network of COX20 has not been carried out. Thus, we used quantitative mass spectrometry to define COX20 interaction partners. Besides COX2 and the expected assembly factors, we identified the uncharacterized human protein TMEM177. Apparently, TMEM177 lacks a clear homolog in yeast. We show that TMEM177 is involved in the COX2 biogenesis pathway. Although TMEM177 is dispensable for cytochrome *c* oxidase activity, its amount is directly linked to COX20 abundance. Since COX2 is stabilized during depletion and overexpression of TMEM177, we suggest that TMEM177 is involved in COX2 stability and turnover.

2. Materials and methods

2.1. Cell culturing, mutagenesis and generation of cell lines

HEK293T Flp-In™ T-REX™ or HEK293 were cultured in DMEM media, supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μg/mL uridine at 37 °C under a 5% CO_2 humidified atmosphere [42]. For inhibition of mitochondrial translation, the medium was supplemented with 50 μg/mL thiamphenicol for two days. Stable FLAG-tag TMEM177 and COX20 expressing cell lines were generated as described previously [43] according to sequences at the NCBI-database: NM_001105198.1 (TMEM177), NM_001312871.1 (COX20). The mutant version of $COX20^{FLAG}$, encoding the amino acid substitution T52P, was generated using the Quickchange™ Lightning Site-Directed Mutagenesis Kit. Transient transfections were performed using GeneJuice® (Novagen) according to manufacturer's instruction. FLAG-constructs were induced with 10 ng/mL doxycycline for 12 h or 72 h prior to harvest. For analysis of cell viability, 0.4% trypan blue (Gibco) was added to the cells collected in PBS. Afterwards, cell numbers were measured directly with a hemocytometer.

2.2. siRNA-mediated knockdown

The following siRNA constructs and concentration were applied: COX20 (5′-GGAGGGUUUAUCUUGGUGA-3′, 33 nM), TMEM177 (5′-GACACUUGUUCCGAAUCAA-3′, 50 nM) (Eurogentec). Transfection reactions were performed on 500,000 cells/25 cm^2 with Lipofectamine RNAiMAX (Invitrogen) in OptiMEM medium according to manufacturer's specification. HEK293T cells were analyzed 72 h after transfection [43].

2.3. In vivo labeling of mitochondrial translation products

Labeling of mitochondrial translation products was performed on 500,000 cells/25 cm^2 [44]. In pulse-chase experiments, cytosolic translation was inhibited with 100 μg/mL anisomycin and mitochondrial translation products labeled with 0.2 mCi/mL [^{35}S]methionine for 2 h. Afterwards, radioactive media was replaced with standard growth media and cells further incubated under standard growth conditions for 3 h or 12 h. In pulse experiments, anisomycin was substituted with 100 μg/mL emetine and cells were pulsed for 1 h. Cells were harvested in 1 mM EDTA/PBS and further analyzed by SDS-PAGE. Signals were detected by Storage Phosphor Screens and a Storm 820 scanner (GE-Healthcare) Signals were quantified with the ImageQuant TL software (GE Healthcare).

2.4. Isolation of mitochondria, fractionation and protein localization analysis

HEK293T cells were collected in 1 mM EDTA/PBS and isolation of mitochondria performed as described [45,46]. For fractionation analyses, samples of the homogenized cells were taken. Crude mitochondria were separated by centrifugation at 11,000*g* for 10 min at 4 °C. To separate ER membranes from the cytosol, the post-mitochondrial supernatant was centrifuged at 100,000*g* for 1 h at 4 °C. Cell equivalents of each fraction were loaded on the gel. To determine the protein localization, isolated mitochondria in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS [morpholinepropanesulfonic acid] pH 7.2) were re-isolated and re-suspended in SEM buffer containing 450 mM KCl and 1% Triton X-100 or their proteins were extracted with 0.1 M Na_2CO_3 buffer, pH 10.8 or pH 11.5 [47]. Then, samples were centrifuged for 30 min at 100,000*g* at 4 °C in a TLA-55 rotor (Beckman Coulter). After addition of TCA, the samples were precipitated and analyzed by SDS-PAGE and Western blotting. Submitochondrial localization was determined by protease protection assay [48]. Mitochondria re-suspended in 10 mM MOPS pH 7.2 were either sonicated, osmotically stabilized in SEM buffer or swollen in EM buffer (1 mM EDTA, 10 mM MOPS pH 7.2). Then, samples were treated with proteinase K for 10 min on ice and reactions were stopped by addition of 1 mM PMSF (Phenylmethylsulfonyl fluoride). Samples were analyzed by SDS-PAGE and Western blotting.

2.5. Antibodies

Primary antibodies were purchased (anti-FLAG and anti-COX20, Sigma-Aldrich; anti-COX2, anti-COX18, anti-COX5B Proteintech Group; anti-TMEM177, Abcam) or raised in rabbit and HRP- or fluorophore-coupled secondary antibodies were employed for detection of antibody-protein complexes an enhanced chemiluminescence system detected by X-ray films or laser scanned on an FLA-9000 (Fujifilm).

2.6. Affinity purification

Protein complexes were purified from isolated mitochondria or whole cell extracts bearing FLAG-tagged proteins or not using anti-FLAG-agarose affinity resin as described previously [43]. For this purpose, mitochondria or cells were solubilized (1 mg/mL) in solubilization buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10% *v*/v Glycerol, 2 mM PMSF, 1 mM EDTA and 1% digitonin) for 30 min on ice. Solubilized material was clarified by centrifugation at maximum speed for 10 min at 4 °C. The supernatant was added to anti-FLAG-agarose affinity resin and incubated for 1 h at 4 °C with shaking. After binding, the beads were washed 10 times with washing buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10% v/v Glycerol, 1 mM PMSF, 1 mM EDTA and 0.3% digitonin) and bound proteins eluted with 0.1 M glycine, pH 2.8, or by competition with FLAG-peptide at 4 °C for 30 min with mild agitation. Subsequently, eluate proteins were analyzed with SDS-PAGE, BN-PAGE or mass spectrometry.

Co-immunoprecipitation of COX20 was carried out following the same procedure. COX20-specific antisera was bound to Protein A-Sepharose using dimethyl pimelimidate as crosslinker as described [49,50].

2.7. SILAC labeling and mass spectrometry

For SILAC experiments [51], cells were grown for five passages in "SILAC" DMEM media as described previously [43]. Mitochondria were isolated from differentially labeled cells, equally pooled, and solubilized. $COX20^{FLAG}$ complex isolation using anti-FLAG-agarose affinity resin was performed as described above. Four independent replicates including label switch were performed.

Proteins of $COX20^{FLAG}$ complexes were acetone-precipitated,