BBA - Bioenergetics 1859 (2018) 244–252

Contents lists available at ScienceDirect

BBA - Bioenergetics

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

COX16 is required for assembly of cytochrome *c* oxidase in human cells and is involved in copper delivery to COX2

Cristina Cerqua[a], Valeria Morbidoni[a], Maria Andrea Desbats[a], Mara Doimo[a,b], Chiara Frasson[c], Sabrina Sacconi[d,e], Maria Cristina Baldoin[a], Geppo Sartori[f], Giuseppe Basso[c], Leonardo Salviati[a,*,1], Eva Trevisson[a,*,1]

[a] Clinical Genetics Unit, Department of Women and Children's Health, University of Padova, Via Giustiniani 3 and IRP Città della Speranza, Corso Stati Uniti 4, Padova, Italy
[b] Department of Medical Biochemistry and Biophysics, Umeå University, SE-901 87, Umeå, Sweden
[c] Hematology-Oncology Laboratory, Department of Women and Children's Health, University of Padova, Via Giustiniani 3, Padova, Italy
[d] CNRS UMR7277, INSERM U1091, IBV - Institute of Biology Valrose, UNS Université Nice Sophia-Antipolis, Faculté de Médecine, Parc Valrose, Nice, CEDEX, France
[e] Centre de référence des Maladies Neuromusculaires, Hôpital Archet 1, 151, Route Saint-Antoine de Ginestière, Nice, France
[f] Department of Biomedical Sciences, University of Padova, Via Ugo Bassi 58B, Padova, Italy

ARTICLE INFO

Keywords:
Cytochrome *c* oxidase
Mitochondrial respiratory chain
Cytochrome *c* oxidase assembly
Copper

ABSTRACT

Cytochrome *c* oxidase (COX), complex IV of the mitochondrial respiratory chain, is comprised of 14 structural subunits, several prosthetic groups and metal cofactors, among which copper. Its biosynthesis involves a number of ancillary proteins, encoded by the COX-assembly genes that are required for the stabilization and membrane insertion of the nascent polypeptides, the synthesis of the prosthetic groups, and the delivery of the metal cofactors, in particular of copper. Recently, a modular model for COX assembly has been proposed, based on the sequential incorporation of different assembly modules formed by specific subunits.

We have cloned and characterized the human homologue of yeast *COX16*. We show that human *COX16* encodes a small mitochondrial transmembrane protein that faces the intermembrane space and is highly expressed in skeletal and cardiac muscle. Its knockdown in *C. elegans* produces COX deficiency, and its ablation in HEK293 cells impairs COX assembly. Interestingly, *COX16* knockout cells retain significant COX activity, suggesting that the function of COX16 is partially redundant.

Analysis of steady-state levels of COX subunits and of assembly intermediates by Blue-Native gels shows a pattern similar to that reported in cells lacking *COX18*, suggesting that COX16 is required for the formation of the COX2 subassembly module. Moreover, COX16 co-immunoprecipitates with COX2. Finally, we found that copper supplementation increases COX activity and restores normal steady state levels of COX subunits in *COX16* knockout cells, indicating that, even in the absence of a canonical copper binding motif, COX16 could be involved in copper delivery to COX2.

1. Introduction

Cytochrome *c* oxidase (COX) (EC 1.9.3.1,) or complex IV, is the terminal enzyme of the mitochondrial respiratory chain (MRC) and it catalyzes the transfer of reducing equivalents from cytochrome *c* to oxygen [1]. The energy produced by this reaction is used to generate the electrochemical gradient that drives ATP synthesis by ATP synthase. COX is active as a dimer; each monomer comprises 14 subunits [2] and a number of prosthetic groups that contribute to redox center formation: two heme groups (*a* and *a3*), three copper ions (two of which are contained in the Cu_A site of COX), a zinc ion and a magnesium ion. The three larger subunits (COX1, COX2 and COX3, encoded by mitochondrial DNA) are embedded in the mitochondrial inner membrane (MIM) and form the catalytic core of the enzyme. The 11 smaller subunits, encoded by nuclear DNA, have regulatory and structural functions.

COX assembly is a complex multi-step process that requires the participation of a large number of assembly factors encoded by nuclear COX-assembly genes. These genes have been identified in yeast, studying respiratory-deficient *pet* mutant strains [3], but only a minority of their human homologues have been thoroughly characterized.

* Corresponding authors at: Clinical Genetics Unit, Department of Pediatrics, University of Padova, Via Giustiniani 3 35128, Padova, Italy.
E-mail addresses: leonardo.salviati@unipd.it (L. Salviati), eva.trevisson@unipd.it (E. Trevisson).
[1] Shared senior authorship.

https://doi.org/10.1016/j.bbabio.2018.01.004
Received 13 September 2017; Received in revised form 23 December 2017; Accepted 17 January 2018

Originally, human COX assembly was thought to be characterized by the sequential incorporation of individual COX subunits, to form the holoenzyme. The process was defined by four steps: early, intermediate, late and last. Recently, a modular model was proposed, based on the sequential incorporation of different assembly modules, formed by specific subunits [4]. COX1, which was originally considered to be the first subunit to be incorporated, was instead found to enter the process after COX4 and COX5A, forming the first module together with the assembly factors COA3, COA1, COX14, CMC1 and SURF1. A second module, formed by the structural subunits COX2, COX5B, COX6C, COX7C and COX8A, was discovered to be incorporated in the same step. Next, COX3 forms the late sub-assembly module together with COX6A, COX6B, and COX7A. Finally, the last module is constituted by NDUFA4 and COX7A2L.

The COX2 module requires several assembly factors for its formation [5]. Among these, COX18 and COX20 are essential for the initial membrane insertion of COX2. Next, SCO1, SCO2 and COA6 promote the insertion of two copper ions [6] (which are delivered to SCO1 by the soluble chaperone COX17 [7]) in the Cu_A site of COX2.

In this work we report the genetic and functional characterization of the human homologue of yeast *COX16*, a gene essential for cytochrome *c* oxidase biogenesis [8].

2. Materials and methods

2.1. Identification and cloning of human COX16

The human homologue of yeast *COX16* (*hCOX16*) was identified as previously described [9,10]. A fragment encompassing the whole coding region of *hCOX16* was amplified from human fibroblasts RNA with primers 5′-GCTGAGATTTGGGAGTCTGC-3′ and 5-CGTGGCCAAC ATTGTTACTTT-3′. PCR conditions were as follows: 94 °C 3 min, 35 cycles at 94 °C 3 min, 55 °C 1 min, 72 °C 45 s and a final extension step of 7 min at 72 °C. Bioinformatic analysis of the protein and of the promoter region was performed as described previously [11].

2.2. RNA isolation and RT-PCR amplification

Total cellular RNAs were extracted from primary human fibroblasts and other cell lines using the TRIzol Reagent (Invitrogen), according to the manufacturer's protocol. Reverse transcription reactions were performed with 1 μg of total RNAs, using random hexamers provided by the manufacturer, and carried out using SuperScript II Reverse Transcriptase (Invitrogen).

2.3. Northern blot

The cloned cDNA was used to synthesize ^{32}P labeled probes, as described [9]. Probes were hybridized at 42 °C in 50% formamide with a pre-made multiple-tissue Northern blot (Clontech) containing 2 μg of poly(A) + RNA from eight human tissues per lane, according to the manufacturer's protocol.

2.4. RACE analysis

To characterize the structure of the different transcripts, we employed a rapid amplification of cDNA ends (RACE), using the Gene Racer kit (Invitrogen), according to the manufacturer's guidelines; the procedure has been detailed elsewhere [9].

The 5′ extremity of *hCOX16* was amplified using primers 266R 5′ TCTTCCCAAGGCCTGGGTCCTCGAA 3′ and GeneRacer-5′ for the first step, and primers 24R 5′ ACGCATCACCGCGGGTGCAAACAT 3′ and GeneRacer-5′ nested for the second step. The 3′ extremity of *hCOX16* transcripts was amplified by two different reactions, using primers 5′ GGGGTTTCACCACGTTAGCCAGGAT 3′ or 5′ CCCAGCTATTCCATCTG TGGATGA 3′and GeneRacer-3′ for the first step, and 5′ TGGCCGAAA GTTAGTTGTTTTGAA 3′ or 5′ TTTGCAATAGCCATTAGGGCATC 3′ and GeneRacer-3′ nested for the second step.

The PCR products were cloned into the pCR4TOPO vector (Invitrogen), and 100 clones were sequenced with vector primers.

2.5. Generation of expression constructs

A HA (hemagglutinin influenza virus)-tagged version of COX16 (HA-COX16) was generated by amplifying the coding region of the gene without the termination codon with the COX16Hind III F primer (5′–CTTCCTAAGCTTATGTTTGCACCCGCGGTGATGCG-3′) and a reverse primer containing the HA sequence, a TGA stop codon and the *Not* I site (5′-CTTCCTGCGGCCGCTCATGCATAGTCTGGTACATCATAAGGG TAAGTTGTCTTAGTCTTAAGGCTTTCTGG-3′). After digestion with the appropriate nucleases, the PCR product was cloned into the pEGFPN1 vector, which was cut similarly in order to remove the GFP sequence.

Human *COX16* coding region was cloned also into the pYES2.1V5HisTOPO vector (Invitrogen). The yeast gene was amplified from *S. cerevisiae* genomic DNA, using primers 5′-CTCCTAAATGTCGT TCAGCGG-3′ and 5′-TTACCAGACATTCTCAGATTC-3′, and cloned into the pYES2.1V5HisTOPO vector.

A *S. cerevisiae–H. sapiens* chimeric ORF, consisting of the 5′region of *yCOX16* encoding the putative mitochondrial import signal and the transmembrane domain, and the remaining 246 bp sequence of *hCOX16* encoding the soluble domain, was obtained by sequential PCR and cloned into the pYES2.1/V5-His TOPO vector. The initial two PCRs were performed with the primers yCOX16F 5′-CTCCTAAATGTCGTTC AGCGG-3′ and 5′-CCTCCAACAATTGTTGCACAAAATGGTAAACC-3′, which amplify *yCOX16* from position −7 to +132, and 5′-TTTGTGC AACAATTGTTGGAGGTTCTTTTGG-3′ and hCOX16R 5′-TGGAATTTCC TTTCCACTTGA-3′, which amplify *hCOX16* from position +76 to +419 (in the 3′UTR). The two PCR products, which have a common 22 bp overlap, were used as templates for a third PCR with primers yCOX16F and hCOX16R, to create the chimeric gene, as described [12]. All constructs were sequenced using vector-specific primers.

Yeast *COX16* (YJL003w) and *SCO1* (YBR037c) genes were cloned into the yeast expression vector pCM189 (primers and PCR conditions are available upon request).

The DNA sequence of a His-tagged version of h*COX16* (*COX16-His*) was amplified by PCR reaction from the pcDNA3.1hygro + vector previously generated in our lab. It was inserted into the pCR™8/GW/ TOPO entry vector (Invitrogen) using the TA-cloning technique, following the manufacturer's protocol. It was then subcloned into the pLentiCMVhygroDEST retroviral transducing vector (Invitrogen) via homologous recombination, using the Gateway LR Clonase II Enzyme Mix Kit (Invitrogen, USA), according to the manufacturer's protocol. Plasmid DNA was isolated using the Plasmid Midi Kit (Qiagen), according to manufacturer's instructions, to obtain clean, free from salt and contaminants DNA, suitable for cell transfection.

2.6. Cell culture and generation of COX16 knockout cells using the CRISPR/Cas9 technology

Human embryonic Kidney (HEK) 293 (Invitrogen) and HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% Fetal Bovine Serum (FBS, scomplemented at 55 °C for 1 h, Gibco), 1% Penicillin/Streptomycin (Gibco) and 1% L-Glutamine (Gibco).

293FT cells (Invitrogen) were grown in DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1% L-Glutamine, 1 mM MEM sodium pyruvate and 500 μg/ml Geneticin.

HEK293 cells were transfected with three pCLIP-All-EFS-Puro vectors containing different CRISPR target sequences of *hCOX16* (TEVH-1107982, TEVH-1175124 and TEVH-1242266, Transomic Technology), using the OMNIfect reagent (Transomic Technology), according to the manufacturer's protocol. After 24 h cells were selected by the addition