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LYRM7/MZM1L is a UQCRFS1 chaperone involved in the last steps of mitochondrial Complex III assembly in human cells

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ABSTRACT

The mammalian Complex III (CIII) assembly process is yet to be completely understood. There is still a lack in understanding of how the structural subunits are put together and which additional factors are involved. Here we describe the identification and characterization of LYRM7, a human protein displaying high sequence homology to the *Saccharomyces cerevisiae* protein Mzm1, which was recently shown as an assembly factor for Rieske Fe–S protein incorporation into the yeast cytochrome bc_1 complex. We conclude that human LYRM7, which we propose to be renamed MZM1L (MZM1-like), works as a human Rieske Fe–S protein (UQCRFS1) chaperone, binding to this subunit within the mitochondrial matrix and stabilizing it prior to its translocation and insertion into the late CIII dimeric intermediate within the mitochondrial inner membrane. Thus, LYRM7/MZM1L is a novel human CIII assembly factor involved in the UQCRFS1 insertion step, which enables formation of the mature and functional CIII enzyme.

1. Introduction

Complex III (CIII) or ubiquinol:cytochrome c oxidoreductase (E.C. 1.10.2.2; the cytochrome bc_1 complex) is the central enzyme of the mitochondrial respiratory chain. It receives electrons from Coenzyme Q, which is reduced mainly by Complex I through NADH-linked substrates and by Complex II through $FADH_2$-linked substrates. CIII then reduces cytochrome c, which transfers the electrons to Complex IV, where molecular oxygen is reduced to water. The oxidation and reduction reactions of CIII are coupled to proton translocation from the mitochondrial matrix to the intermembrane space by the so-called Q cycle [1], contributing to the membrane potential necessary for ATP synthesis.

Mammalian CIII possesses a symmetrical dimeric structure in which each "monomer" is composed of 11 different subunits [2,3], three of which are the catalytic subunits: MT-CYB (cytochrome b, the only mtDNA-encoded subunit), CYC1 (cytochrome c_1) and UQCRFS1 (Rieske Fe–S protein).

The CIII assembly process has mainly been studied by using the yeast *S. cerevisiae* as a model, taking advantage of this facultative anaerobic organism and its ability to survive on fermentative substrates when its CIII is non-functional. By analyzing the composition of the sub-complexes present in different yeast deletion strains, a model involving a multi-step process and the formation of different assembly intermediates has been described (reviewed in [4]). In addition, several yeast proteins are known to assist the process by acting in different parts of the assembly pathway [4]. By means of putting all subunits together except the Rieske Fe–S protein (Rip1) and the smallest subunit Qcr10, a considerably stable non-functional "late core" subcomplex, or pre-$CIII_2$ intermediate, is formed. The assembly of the complex is completed when Rip1 is inserted in the last step, followed by Qcr10 [5].

As for all nuclear-encoded mitochondrial proteins, yeast Rip1 is synthesized in the cytoplasm and then imported inside mitochondria. Rip1 is transported completely into the matrix to receive the 2Fe–2S cluster cofactor necessary for its function, and the protein is proteolytically processed in two steps [6]. Differing from yeast, UQCRFS1 in mammals is processed in a single step, and what was originally the pre-sequence is retained as a structural subunit [7]. Translocation of the Rieske Fe–S protein from the matrix to the mitochondrial inner membrane for its insertion into CIII is mediated by the AAA-ATPase Bcs1 [8]. The human ortholog, BCS1L, seems to perform the same function as in yeast, as demonstrated by analysis of CIII assembly in patients carrying deleterious mutations in the *BCS1L* gene [9–11], in which the pre-$CIII_2$ late intermediate lacking UQCRFS1 is accumulated.

Recently, a yeast protein involved in this last Rip1 insertion step has been described [12,13]. Lack of this protein, Mzm1, is associated with a defect in CIII maturation and reduced CIII activity, as well as very low Rip1 steady-state levels. Furthermore, Mzm1 was demonstrated to

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interact with Rip1 within the mitochondrial matrix to stabilize it, preventing its proteolytic degradation or its aggregation under conditions in which Rip1 could not be incorporated into CIII [13,14].

Here, we report the finding and functional characterization of LYRM7, a human protein showing high amino acid sequence homology to the yeast Mzm1. Our results point out that LYRM7 is the MZM1-like (MZM1L) protein, i.e., acting as a Rieske Fe–S protein chaperone in human cells that binds to the subunit within the matrix in a step prior to its insertion into the late pre-$CIII_2$ intermediate. Thus, LYRM7/MZM1L appears to be an assembly factor for the late stage of CIII assembly in humans.

2. Materials and methods

2.1. Cell lines and cell culture

Human primary and immortalized skin fibroblasts, HEK 293T and HeLa cells were grown at 37 °C in a 5% CO_2 atmosphere in high-glucose plus glutamine and sodium pyruvate DMEM medium (Gibco-Life Technologies) supplemented with 10% fetal bovine serum (FBS from PAN-Biotech), 1× penicillin–streptomycin (Gibco-Life Technologies) and 50 μg/ml Uridine (in the case of CIII-deficient cell lines). For the puromycin-resistant cells, a final concentration of 1 μg/ml in the medium was used. Mouse L929 fibroblasts were cultured in the same conditions except for the final FBS concentration, which was 5%.

Primary human skin fibroblasts were immortalized by lentiviral transduction using the pLox-Ttag-ires-TK vector (Tronolab) [15].

2.2. Yeast growth assay

S. cerevisiae expression vectors were constructed to bear the coding sequences for: 1) the human gene *LYRM7* (transcript 1; see Section 2.3), followed by 6 histidine repeats and a single Myc tag, 2) the yeast gene *MZM1*, followed by a single Myc tag and 6 histidine repeats, and 3) the yeast gene *SDH6*, followed by 6 histidine repeats and a single Myc tag. These coding sequences were cloned into a common high-copy-number pRS426 plasmid bearing a *URA3* selection marker and expressed under the control of the yeast *MET25* promoter and *CYC1* terminator sequences [16]. These plasmids as well as an empty vector control were transformed into wild-type and Δ*mzm1* deletion yeast strains of the BY4741 genetic background via a variation of the lithium acetate procedure [17]. Transformed cells were grown on selective plates containing Brent supplement mixture lacking uracil (Sunrise Science Products, San Diego, CA) plus 2% glucose, and colonies were inoculated into 4-ml cultures of the same media, grown overnight, and spotted onto selective plates containing either 2% glucose or 2% glycerol/2% lactate at equivalent optical densities (at 600 nm) in a 10-fold serial dilution. Plates were incubated at 30 °C or 37 °C and photographed after ~48 h (glucose) and ~144 h (glycerol-lactate) of growth.

2.3. LYRM7 constructs

cDNA was obtained by using the GoScript reverse transcription system (Promega), using total RNA extracted from cultured cells with TRIzol reagent (Invitrogen). PCR products were produced using cDNA as the template with specific primers: hLYRM7-MluI-Fw: 5′-CTTTACGCGTCAGTCTTTGATTGGTTGCTG-3′ and hLYRM7-SalI-Rv: 5′-CCCCGTCGACCTTGTTGTATTCTAGAAAAC-3′; mLyrm7-MluI-Fw: 5′-CTTTACGCGTGGGGAGCCATGGGTCAG-3′ and mLyrm7-SalI-Rv: 5′-CCCTGTCGACAGAGATGGGTTTATCCTGG-3′. The obtained PCR products were cloned into the pCR2.1 TA cloning system (Invitrogen). Sequence checked clones containing the two insert variants (LYRM7-001 and LYRM7-003) were used as templates for the amplification to add the HA tag at the C-terminus of the putative protein products, using the same hLYRM7-MluI-Fw primer and hLYRM7-001-HA-SalI-Rv: 5′-CCCGTCGACTCAAGCGTAATCTGGAACATCGTATGGGTATTGCTTCTGAGTTGGTGCATC-3′ or hLYRM7-003-HA-SalI-Rv: 5′-CCCGTCGACTCAAGCGTAATCTGGAACATCGTATGGGTACAAGAAGGTCTTTCCTAGGG-3′. The PCR products were cloned into the pCR2.1 TA cloning system (Invitrogen). Inserts with the correct sequences were subsequently cloned into a lentiviral expression vector derived from pWPXLd (Tronolab), in which the GFP sequence was substituted by a puromycin resistance cassette (pWPXLd-ires-$Puro^R$).

2.4. Lentiviral transduction

Lentiviral particles containing the LYRM7-001-HA/pWPXLd-ires-$Puro^R$, LYRM7-003-HA/pWPXLd-ires-$Puro^R$ or the empty pWPXLd-ires-$Puro^R$ vectors were generated in HEK 293T packaging cells, and HeLa cells were transduced with the former as described [18]. Twenty-four hours after transduction, cells were selected for puromycin resistance (Section 2.1).

2.5. Isolation and subfractionation of mitochondria

Mitochondrial preparations from transduced HeLa cells were obtained as described [19]. For subfractionation of mitochondria, to separate the soluble and membranous fractions, freshly isolated mitochondria were sonicated three times and then centrifuged at 100,000 ×*g* for 30 minutes at 4 °C to separate the supernatant containing the soluble proteins and the membrane pellet [20]. To split the peripherally bound from the integral membrane proteins in the pellet from the previous step, the samples were resuspended in a buffer containing 0.1 M Na_2CO_3, pH 10.5, 0.25 M sucrose and 0.2 mM EDTA; incubated for 30 min on ice and then centrifuged at 100,000 ×*g* for 30 minutes at 4 °C, to separate the pellet from the supernatant [21].

2.6. Immunoprecipitation

Approximately 500 μg of mitochondrial protein isolated from HeLa cells, either transduced with the empty pWPXLd-ires-$Puro^R$ vector or overexpressing LYRM7-001-HA (MZM1L-HA), were lysed in PBS with 140 mM NaCl, 1% n-Dodecyl β-D-maltoside (DDM) and protease inhibitor cocktail (Sigma), during 30 minutes on ice. The lysate was cleared by centrifugation at 20,000 ×*g* for 30 minutes and divided into three aliquots, which were each incubated with 1.5 μg of high affinity anti-HA antibody (Roche), anti-Rieske protein antibody (Molecular Probes-Invitrogen) or mouse serum IgGs (Sigma) for 5 hours at 4 °C. Immunoprecipitation was achieved by adding pre-washed Protein G-Sepharose 4B beads (Invitrogen) and incubating an additional 2 hours at 4 °C. The co-immunoprecipitates were washed three times in astringent conditions (PBS with 400 mM NaCl, 0.1% DDM and protease inhibitor cocktail) and once in low salt conditions (PBS with 140 mM NaCl, 0.1% DDM and protease inhibitor cocktail). Proteins were finally eluted from the beads with 2× Laemmli Sample Buffer and heated at 95 °C for 5 minutes.

2.7. Protein electrophoresis, Western blot and immunodetection

Total protein extracts from cultured cells or fractions were resolved under denaturing conditions using 16.5% polyacrylamide Tricine-SDS-PAGE [22] or standard 15% polyacrylamide SDS-PAGE.

Blue-Native Gel Electrophoresis (BNGE) was performed using mitoplast samples prepared by lysing cells in the presence of digitonin, followed by a final solubilization with 1% DDM as described [23]. The complexes were resolved in 5–13% gradient polyacrylamide native gels [24]. For the second denaturing dimension, 16.5% polyacrylamide Tricine-SDS-PAGE preceded by a 10% polyacrylamide stacking gel was used [9].

The gels were electroblotted to PVDF membranes, and the immobilized proteins were immunodetected using specific antibodies as indicated in each case. Anti-Core 1 (CIII subunit 1), anti-Core 2 (CIII subunit 2), anti-COI (Complex IV subunit I) and Mitoprofile (Total