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Role of the mitochondrial ATP synthase central stalk subunits γ and δ in the activity and assembly of the mammalian enzyme

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ABSTRACT

The central stalk of mitochondrial ATP synthase consists of subunits γ, δ, and ε, and along with the membraneous subunit c oligomer constitutes the rotor domain of the enzyme. Our previous studies showed that mutation or deficiency of ε subunit markedly decreased the content of ATP synthase, which was otherwise functionaly and structuraly normal. Interestingly, it led to accumulation of subunit c aggregates, suggesting the role of the ε subunit in assembly of individual enzyme domains. In the present study we focused on the role of subunits γ and δ. Using shRNA knockdown in human HEK293 cells, the protein levels of γ and δ were decreased to 30% and 10% of control levels, respectively. The content of the assembled ATP synthase decreased in accordance with the levels of the silenced subunits, which was also the case for most structural subunits. In contrast, the hydrophobic c subunit was increased to 130% or 180%, respectively and most of it was detected as aggregates of 150–400 kDa by 2D PAGE. In addition the IF_1 protein was upregulated to 195% and 300% of control levels. Both γ and δ subunits silenced cells displayed decreased ATP synthase function - lowered rate of ADP-stimulated respiration, a two-fold increased sensitivity of respiration to inhibitor oligomycin, and impaired utilization of mitochondrial membrane potential for ADP phosphorylation. In summary, similar phenotype of γ, δ and ε subunit deficiencies suggest uniform requirement for assembled central stalk as driver of the c-oligomer attachment in the assembly process of mammalian ATP synthase.

1. Introduction

The life of the eukaryotic cell fully depends on mitochondrial ATP synthase - not only does it provide > 90% of ATP in mammalian organisms, but recent studies also established the role of ATP synthase dimers in cristae architecture formation or in the mitochondrial permeability transition phenomenon (reviewed in [1]). Furthermore, its ability to switch into ATP hydrolysis mode may help maintaining membrane potential and integrity of mitochondria under hypoxic conditions, while involvement in permeability transition pore may link the ATP synthase to mitoptosis and cell death [2]. ATP synthase abundance and its ATP hydrolytic and proton conductance capabilities underline the need for tight regulation of the enzyme.

The role of central stalk subunits in ATP synthase biogenesis and function was repeatedly studied in yeast *S. cerevisiae* using disruptions of corresponding genes *ATP3*, *ATP16*, and *ATP15*, encoding subunits γ, δ, and ε, respectively. Loss of any of these subunits causes inability to grow on non-fermentable carbon source, indicating essential role of central stalk in the coupling of proton translocation to ATP synthesis [3]. In addition, loss of subunits γ and δ causes severe instability of mitochondrial genome resulting in formation of 100% ρ^-/ρ^0 colonies. Deletion of the ε subunit has slightly less dramatic phenotype with about 70% petite colonies [4,5]. The petite phenotype, however, was prevented by F_o-binding inhibitor oligomycin, disruption of *ATP1* or *ATP2* genes encoding F_1 subunits α and β, respectively, or *ATP4* (peripheral stalk subunit b) mutations [4]. These findings indicated that incomplete ATP synthase complexes consisting of catalytic hexamer, peripheral stalk, and proton channel could be assembled in *S. cerevisiae*, despite the absence of central stalk subunit(s). Formation of petite colonies and subsequent absence of mtDNA-encoded constituents of proton channel Atp6 and Atp9, then serves as a survival strategy preventing lethal loss of proton motive force through these uncoupled ATP synthase subcomplexes [4,6]. On the other hand, the formation of aberrant uncoupled ATP synthase assemblies was not implicated upon ε

Abbreviations: F_1, catalytic part of ATP synthase; F_o, membrane embedded part of ATP synthase; IF_1, F_1 inhibitor protein; F_1 α, F_1 β, F_1 γ, F_1 δ, F_1 ε - F_1 subunits α, β, γ, δ, and ε; F6, ATP synthase subunit coupling factor 6; OSCP, ATP synthase subunit oligomycin sensitivity conferring protein; F_o a, F_o d, F_o c, F_o subunits a, d and c; $\Delta\Psi_M$, membrane potential of mitochondrial membrane

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subunit deletion in different yeast organism *K. lactis* [7]. The same holds true for mammalian ATP synthase - our previous studies on two models of subunit ε dysfunction, caused by rare missense Tyr12Cys mutation of *ATP5E* gene [8] or by shRNA silencing in HEK293 cells [9], showed only profound decrease of ATP synthase holoenzyme level with notable absence of any F_1 subassemblies. The only accumulated ATP synthase subcomplex contained subunit F_o-c but none of the other tested subunits, including F_o a. Unchanged capacity of passive proton leak detected by respirometry and membrane potential measurements indicated that this subunit c subcomplex was not able to conduct protons [9]. These findings indicated strict control during the process of mammalian ATP synthase assembly and efficient elimination of subcomplexes capable of unregulated proton translocation or ATP hydrolysis. In the present study, we extend our previous work on subunit ε downregulation to the two remaining central stalk subunits γ and δ. We show that silencing expression of these subunits in HEK293 resulted in identical phenotype as ε deficiency, i.e. decreased content of ATP synthase, accumulation of F_o c hydrophobic subunit representing the only detected ATP synthase subcomplex and unchanged proton leak. Our findings thus indicate uniform position and essential role of central stalk subunits in the assembly of mammalian ATP synthase.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney 293 cells (HEK293, ATCC) were grown at 37 °C in a 5% (v/v) CO_2 atmosphere in high-glucose Dulbecco's modified Eagle's medium (PAA) supplemented with 10% (v/v) fetal calf serum (Sigma).

2.2. Silencing of ATP synthase subunits expression

For the silencing of subunit γ of ATP synthase (gene ATP5C1, transcript NM_005174) we tested six individual miR-30-based shRNAs (shRNAmirs) targeting both alternative transcripts NM_005174 and NM_001001973 of human ATP5C1 gene (V3LHS_345497, V3LHS_345498, V3LHS_345500, V2LHS_48722, V2LHS_48723, V3LHS_48724, all cloned into pGIPZ™ plasmid, Open Biosystems). Subunit δ (gene ATP5D, transcript NM_001001975.1) was targeted by four individual shRNAs (V3LHS_388106, V3LHS_388107, V3LHS_388110, V3LHS_388111, Open Biosystems). Plasmid DNA was isolated by an endotoxin-free kit (Qiagen) and transfected into HEK293 cells using Arrest-in kit (Bioxys). At 48 h after transfection the cells were exposed to culture medium containing 1.5 μg/ml puromycin (Sigma-Aldrich) and antibiotic-resistant colonies were selected over a period of three weeks. The efficiency of silencing was assessed by quantification of the respective protein by Western blot. Two clones with most efficient silencing of each gene were selected for subsequent experiments. Clones of HEK293 cells transfected with empty vector were used as controls (non-silenced cells, NS).

2.3. Electrophoretic analyses

SDS-Tricine polyacrylamide gel electrophoresis (SDS-PAGE) [10] was performed on 10% (w/v) polyacrylamide slab minigels. The samples of whole cell lysates were incubated for 20 min at 40°C in 2% (v/v) mercaptoethanol, 4% (w/v) SDS, 10 mM Tris-HCl, 10% (v/v) glycerol.

For native electrophoresis of intact ATP synthase complexes and subassemblies, isolated membrane fractions [11] of control and silenced cells were solubilized by digitonin (2 g detergent/g of protein) or dodecyl maltoside (n-dodecyl-β-D-maltoside; 1 g detergent/g of protein). Solubilized proteins were resolved using the high-resolution clear native electrophoresis (hrCNE1) system [11] and ATP synthase complexes were detected by activity staining or using Western blotting techniques.

For two-dimensional (2D) analysis, the individual stripes of the first dimension hrCNE1 gel were incubated for 1 h in 1% (w/v) SDS and 1% (v/v) mercaptoethanol and then subjected to SDS-PAGE in the second dimension.

2.4. Western blot analysis

Gels were blotted on to PVDF membrane (Millipore) by semi-dry electrotransfer (1 h at 0.8 mA/cm^2). Blocked membranes (5% (v/v) milk in TBS) were incubated in TBS, 0.01% (v/v) Tween 20 with the following primary antibodies – polyclonal antibodies against F_o a (1:500 [12]), F_o c (1:1000 [13]), F_1 γ (1:2000; 10910-1-AP, Proteintech), and F_1 δ (1:1000; 14893-1-AP, Proteintech), monoclonal antibodies against F_1 α (1:1000; MS502, Mitosciences), F_1 β (1:2000; MS503, Mitosciences), F_1 ε (1:500; H00000514-M01, Abnova), F_o d (1:1000; MS504, Mitosciences), F_o OSCP (1:1000; MS505, Mitosciences), F_o F6 (1:1000; MS508, Mitosciences), COX4-1 (1:1000; MS408, Mitosciences), SDH70 (1:10000, MS204, Mitosciences), and actin (1:1000, MSA03, Mitosciences), and with fluorescent secondary antibodies (goat anti-mouse IgG, 1:3000, Alexa Fluor 680 A-21058 or goat anti-rabbit IgG, 1:3000, Alexa Fluor 680 A-21109, Molecular Probes). The fluorescence was detected on an Odyssey system (LI-COR) and the signal was quantified using Aida 3.21 Image Analyzer software (Raytest).

2.5. Respiration measurements

Respiration was measured at 30 °C by an Oxygraph-2k (Oroboros). Freshly harvested cells were suspended in a KCl medium (80 mM KCl, 10 mM Tris/HCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate, pH 7.4) and permeabilized with digitonin (0.05 g/g of protein). Substrates and inhibitors were used at following concentrations: 10 mM glutamate, 3 mM malate, 10 mM succinate, 1.5 mM ADP, 5–200 nM oligomycin, 100–200 nM FCCP, 1 μM rotenone, 1 μM antimycin A, 2 mM ascorbate, 1 mM TMPD. Oxygen consumption was expressed in pmol oxygen·s^{-1}·mg protein^{-1}.

2.6. Mitochondrial membrane potential $\Delta\Psi_M$ measurements

$\Delta\Psi_M$ was measured with TPP$^+$-selective electrode in 1 ml of KCl medium as described in [14]. Freshly harvested cells (2.5 mg protein/ml) were permeabilized with digitonin (0.05 g/g protein) and the following substrates and inhibitors were used: 10 mM succinate, 10 mM glutamate, 3 mM malate, 1.5 mM ADP, 1 μM oligomycin and 1 μM FCCP. The acquired data were plotted as pTPP, i.e. natural logarithm of TPP$^+$ concentration (μM).

3. Results

3.1. Decreased content of structurally normal ATP synthase

The impact of stalk subunits knockdown on the content and structural composition of mitochondrial ATP synthase was first evaluated by the quantification of steady state levels of individual enzyme subunits using SDS/PAGE Western blot (Fig. 1). shRNA knockdown of the ATP synthase subunit F_1 γ decreased its steady state level to 35% of control (Fig. 1A, C). Similarly, the content of other five tested ATP synthase subunits – F_1 α, F_1 β, OSCP, F6, and F_o a ranged between 30 and 40% of control levels (Fig. 1A, C). In contrast, the level of subunit F_o c was increased to about 130%, and the amount of the protein inhibitor of ATP synthase – IF_1 was almost two-fold increased (Fig. 1A, C). The content of representative subunits of respiratory chain complexes II (SDH70) and IV (COX4-1) did not differ between knockdown and control cell lines. The shRNA knockdown of F_1 subunit δ was even more efficient than in case of subunit γ, the residual level of δ protein was a mere 10% of control (Fig. 1B, C). Following similar pattern as the γ

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