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Knockdown of F_1 epsilon subunit decreases mitochondrial content of ATP synthase and leads to accumulation of subunit c

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ABSTRACT

The subunit ε of mitochondrial ATP synthase is the only F_1 subunit without a homolog in bacteria and chloroplasts and represents the least characterized F_1 subunit of the mammalian enzyme. Silencing of the *ATP5E* gene in HEK293 cells resulted in downregulation of the activity and content of the mitochondrial ATP synthase complex and of ADP-stimulated respiration to approximately 40% of the control. The decreased content of the ε subunit was paralleled by a decrease in the F_1 subunits α and β and in the F_o subunits a and d while the content of the subunit c was not affected. The subunit c was present in the full-size ATP synthase complex and in subcomplexes of 200–400 kDa that neither contained the F_1 subunits, nor the F_o subunits. The results indicate that the ε subunit is essential for the assembly of F_1 and plays an important role in the incorporation of the hydrophobic subunit c into the F_1-c oligomer rotor of the mitochondrial ATP synthase complex.

1. Introduction

The mammalian ATP synthase (F_oF_1 ATPase) is a heterooligomeric complex of ~650 kDa localized in the inner mitochondrial membrane. It consists of at least 16 different types of subunits [1,2]. Six of them (α, β, γ, δ, ε and inhibitor protein IF_1) form the F_1-catalytic domain on the matrix side of the membrane. The remaining ten subunits (a, b, c, d, e, f, g, OSCP, A6L, F6), two of which (a and A6L) are encoded by mitochondrial DNA (mtDNA) [3], comprise the membrane-embedded F_o portion, functioning as a proton channel, and two stalks connecting the F_1 and F_o domains [4–7]. Two additional proteins MLQ and AGP are possibly involved in the dimerization of ATP synthase [8], and the enzyme function can be modulated by the coupling factor B [9].

The three largest subunits of the F_1 catalytic part of ATP synthase — α, β and γ — exert a varying degree of homology among ATP synthases from mitochondria, chloroplast and bacteria, while the mammalian subunit δ corresponds to the ε subunit in the bacterial enzyme [1]. The only F_1 subunit that does not have a homolog in bacteria and chloroplasts is the ε subunit [1], which is the smallest and functionally the least characterized mitochondrial F_1 subunit. The mammalian ε subunit [10] encoded by the *ATP5E* gene is a 51AA protein of 5.8 kDa that lacks a cleavable import sequence. It exerts a high degree of homology with the slightly larger yeast ε of 6.6 kDa, which is encoded by the *ATP15* gene, and consists of 62 AA in *S. cerevisiae* [11]. As revealed by complementation experiments, the yeast and mammalian ε are structurally and functionally equivalent [12].

The F_1 subunits γ, δ and ε together with the subunit c oligomer form the rotor of ATP synthase [13]. The subunit ε was shown to form heterodimers with the subunit δ [14,15] and presumably also makes contacts with F_o. As revealed by crystallographic studies [5,16], the mitochondrial ε subunit is located in the protruding part of the central stalk and it has a hairpin (helix–loop–helix) structure. It maintains contact with the γ and δ subunits and is expected to be involved in the stability of the foot of the central stalk facing the c subunit oligomer. The C-terminus of ε subunit forms an extension of the β-sheet of γ subunit and the N-terminal region of ε subunit is located in a shallow cleft of δ subunit [5].

The involvement of ε subunit in the ATP synthase biogenesis and function was repeatedly studied in yeast by means of disruption of the *ATP15* gene. The absence of ε subunit in *S. cerevisiae* resulted in no detectable oligomycin-sensitive ATPase activity, decreased content of γ, δ and F_o subunits in immunoprecipitated ATP synthase and F_1 instability. High proton leak, which was shown to be sensitive to oligomycin, indicated a conformationally changed F_o [17]. Also, disruption of the *ATP15* gene in *K. lactis* resulted in a complete elimination of F_1-ATPase activity, suggesting that the ε subunit may have an important role in the formation of the F_1 catalytic sector of eukaryotic ATP synthase [18]. In contrast, if the null mutations of F_1 subunits α, β, γ, δ and ε were made in *S. cerevisiae*, mutations in all but the ε subunit gene were unable to grow on a nonfermentable carbon source indicating that ε is not an essential component of the ATP synthase [11].

Abbreviations: DDM, dodecyl maltoside; F_1, catalytic part of ATP synthase; F_o, membrane-embedded part of ATP synthase

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With the aim to investigate the functional role of ε subunit in the biogenesis and formation of mammalian ATP synthase complex, we have downregulated the expression of *ATP5E* gene in HEK293 cells by means of RNA interference (RNAi). We have found that the inhibition of ε subunit biosynthesis has a pronounced effect on the mitochondrial content and activity of ATP synthase and leads to a relative accumulation of subunit c. Our results demonstrate the essential role of subunit ε in the assembly of F_1 and the incorporation of hydrophobic subunit c into the F_1-c oligomer rotor structure of mitochondrial ATP synthase in higher eukaryotes.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney 293 cells (HEK293 from 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 (PAA). Cell transfections were carried out with a Nucleofector™ device (Amaxa) using the HEK293 cell-specific transfection kit.

2.2. RNAi

For the silencing of subunit ε of ATP synthase we used two miR-30-based shRNAs (shRNAmirs) shE1 (TGCTGTTGACAGTGAGCGAACAATGTCAATAAATTGAAATTAGTGAAGCCACAGATGTAATTTCAATTTATTGACATTGTCTGCCTACTGCCTCGGA) and shE2 (TGCTGTTGACAGTGAGCGAACATGTTATGGCAGATTGAAATAGTGAAGCCACAGATGTATTTCAATCTGCCATAACATGTGTGCCTACTGCCTCGGA) targeted to the coding sequence of human *ATP5E* gene, which were cloned to plasmid pGIPZ™ (V2LHS-77373 and V2LHS-773734, Open Biosystems). Plasmid DNA was isolated by an endotoxin-free kit (Qiagen) and HEK293 cells were transfected with the shE1 or shE2 shRNA constructs or with the non-silencing, empty vector (negative control, NS cells). At 48 h after the transfection the cells were split into culture medium containing 1.5 μg/ml puromycin (Sigma-Aldrich) and antibiotic-resistant colonies were selected over a period of three weeks.

ATP5E mRNA and *18S* RNA levels were determined in the transfected cells by QT RT-PCR. The total RNA was isolated with TRIzol reagent (Invitrogen) and cDNA was synthesized with SuperScript III reverse transcriptase using random primers (Invitrogen). PCR was performed on the LightCycler 480 instrument (Roche Diagnostics) with a SYBR Green Master kit (Qiagen) using *ATP5E* (F: 5′-GATGCACTGAAGACAGAATTCAAAG-3′, R: 5′-GCTGCCAGAAGTCTTCTCAGC-3′) and *18S* (F: 5′-ATCAGGGTTCGATTC CGGAG-3′, R: 5′-TTGGATGTGGTAGCCGTTTCT-3′) primers.

2.3. Isolation of mitochondria

Cells (~90% confluent) were harvested with 0.05% trypsin and 0.02% EDTA and washed twice in phosphate-buffered saline (PBS, 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.20 g/l KH_2PO_4). Mitochondria were isolated by a method utilizing hypotonic shock cell disruption [19]. To avoid proteolytic degradation, isolation medium (250 mM sucrose, 40 mM KCl, 20 mM Tris–HCl, 2 mM EGTA, pH 7.6) was supplemented with protease inhibitor cocktail (Sigma P8340). The protein content was measured by the Bio-Rad Protein Essay (Bio-Rad Laboratories), using BSA as a standard. The isolated mitochondria were stored at −70 °C.

2.4. Electrophoresis

Blue-Native polyacrylamide gel electrophoresis (BN-PAGE) [20] was performed on a 6–15% polyacrylamide gradient minigels (Mini Protean, Bio-Rad). Mitochondria were solubilized with dodecyl maltoside (DDM, 2 g/g protein) for 20 min on ice in 1.75 M aminocaproic acid, 2 mM EDTA and 75 mM Bis–tris (pH 7.0). Samples were centrifuged for 20 min at 26000 × *g*, Serva Blue G (0.1 g/g detergent) was added to supernatants and the electrophoresis was run at 45 V for 30 min and then at 90 V.

SDS-Tricine polyacrylamide gel electrophoresis (SDS-PAGE) [21] was performed on 10% (w/v) polyacrylamide slab minigels. The samples 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 two-dimensional (2D) analysis, the stripes of the first dimension BN-PAGE 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 [21].

2.5. 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% (w/v) non-fat dry milk in PBS) were incubated in PBS, 0.01% (v/v) Tween 20 with the following primary antibodies — polyclonal antibodies against F_o-a (1:500 [22]) and F_o-c (1:1000 [23]), monoclonal antibodies against F_1-α (1:1000, MS502, MitoSciences), F_1-β (1:2000, MS503, MitoSciences), F_1-ε (1:5000, Abnova), F_o-d (1:100; Molecular Probes), SDH70 (1:10000, MS204, Mitosciences), Core 2 (1:1000, MS304, Mitosciences) and porin (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 Analyser software.

2.6. ATPase assay

The ATP synthase hydrolytic activity was measured in ATP-regenerating system as described by [24]. Digitonin (0.05 g/g protein) permeabilized cells were incubated in a medium containing 40 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 10 mM KCl, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 1 μM rotenone, 3 μM FCCP, 0.1% (w/v) BSA, 5 U of pyruvate kinase, 5 U of lactate dehydrogenase for 2 min. The reaction was started by addition of 1 mM ATP. The sensitivity to aurovertin or oligomycin was determined by parallel measurements in the presence of 2 μM inhibitor.

2.7. 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 (0.1 g/g of protein). Respiration was measured using 10 mM glutamate, 3 mM malate, 1.5 mM ADP, 1 μM oligomycin, 1 μM FCCP and 1 μM antimycin A. Oxygen consumption was expressed in pmol oxygen s^{-1} mg $protein^{-1}$.

2.8. 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 [25]. 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 membrane potential was plotted as pTPP, *i.e.* natural logarithm of TPP^+ concentration (μM).

3. Results

3.1. Downregulation of ATP5E gene decreases the content and activity of ATP synthase

Transfections of HEK293 cells with miR-30-based shRNAs (shE1, shE2) targeted to the *ATP5E* gene encoding the ε subunit of ATP synthase were followed by puromycin selection and resulted in three shE2 stable lines that showed a variable decrease of *ATP5E* mRNA levels relative to 18S RNA. These shEa, shEb and shEc exhibited an *ATP5E* mRNA level of 16%, 47% and 29%, respectively, compared with the parental HEK293 cells transfected with empty vector (NS cells). The cell lines exerted normal viability under standard cultivation conditions. There was no significant difference in cell growth rate between the silenced and control cell lines.

The cell lines were analyzed for the content and activity of mitochondrial ATP synthase as well as the function of mitochondrial respiratory chain. Quantification of the cellular content of respiratory chain enzymes by SDS-PAGE and WB showed in all silenced cell lines normal content of complexes II and III, but decreased content of complex V — ATP synthase (Fig. 1A), indicating that the specific knockdown of *ATP5E* gene expression affected selectively the biogenesis of ATP synthase complex. Based on the immunodetection with the antibody to F_1 subunit β, the content of ATP synthase showed a decrease of 60–70%. The same result was obtained with the antibody to the α subunit (not shown). This was confirmed by analysis of ATP synthase at native conditions in dodecyl maltoside-solubilized proteins from isolated mitochondria using BN-PAGE and WB (Fig. 1B). In comparison with the controls (the original HEK293 and NS cells), the *ATP5E*-silenced cell lines contained reduced amounts of assembled ATP synthase complex, which, however, retained the same mobility as the ATP synthase complex from control cells corresponding to about 650 kDa. Control cells contained a small amount of F_1 subcomplex of ~370 kDa, which was not detected in silenced cell lines. The quantification of WB data from BN-PAGE revealed also a 60–70% reduction of ATP synthase complex in the *ATP5E*-silenced cell lines.

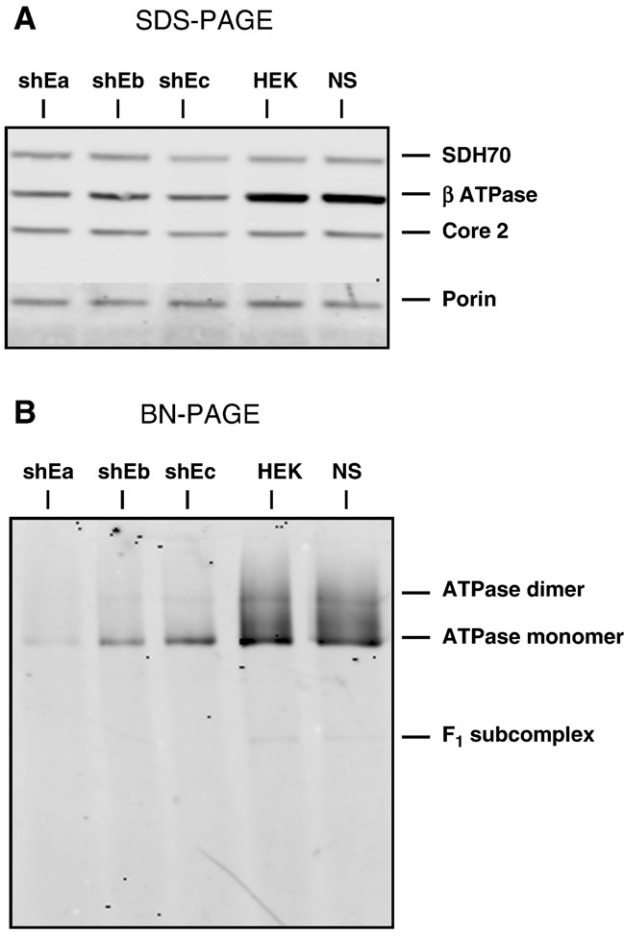

Fig. 1. Selective reduction of ATP synthase in *ATP5E*-silenced cell. (A) Isolated mitochondria (10 μg protein aliquots) from control (HEK293 and HEK293 transfected with empty vector (NS)) and *ATP5E*-silenced (shE2a, shE2b, shE2c) cells were analyzed by SDS-PAGE and WB with antibodies to ATP synthase (β) and to respiratory chain complexes II (SDH70) and III (Core 2). (B) DDM-solubilized (2 g/g protein) mitochondrial proteins (15 μg protein aliquots) from control (HEK293, NS) and *ATP5E*-silenced (shE2a, shE2b, shE2c) cells were analyzed by BN-PAGE and WB using antibody to ATP synthase β subunit.

Furthermore, the *ATP5E*-silenced cell lines had a low ATP synthase hydrolytic activity compared with the control HEK293 and NS cells. Oligomycin-sensitive ATP hydrolysis showed a 54–64% decrease of activity and aurovertin-sensitive ATP hydrolysis was 64–68% decreased in comparison with the control cells. The activity measurements data corresponded well with the electrophoretic analysis. The same results obtained with F_1-interacting aurovertin and F_o-interacting oligomycin indicated further that all remaining ATP hydrolytic activity was due to complete ATP synthase complexes with unaltered F_1–F_o interaction and not due to a presence of free and active F_1-ATPase molecules.

3.2. Downregulation of ATP5E gene decreases mitochondrial ATP production but does not uncouple oxidative phosphorylation

The functional effects of *ATP5E* silencing on mitochondrial energy conversion were analyzed by mitochondrial respiration in digitonin-permeabilized cells. As shown in Fig. 2A, mitochondria in the *ATP5E*-silenced cells were tightly coupled at state 4, but ADP-stimulated respiration was significantly lower although these cells had comparable respiratory capacity after uncoupling with FCCP (state 3 uncoupled) when compared with the control NS (Fig. 2A) or HEK293 (not shown) cells. The ADP-stimulated respiration in the *ATP5E*-silenced cells was fully sensitive to oligomycin. Direct measurements of mitochondrial membrane potential $\Delta\Psi_m$ with TPP^+-selective electrode (Fig. 2B) revealed comparable state 4 values of $\Delta\Psi_m$ in both control and *ATP5E*-silenced cells. In fact, an even higher state 4 value was found in the *ATP5E*-silenced cells (ΔpTPP, i.e. the difference of membrane potential with respect to the pTPP value with FCCP, was 0.47 and 0.64 in the control and silenced cells, respectively). Addition of ADP led to a much smaller decrease of $\Delta\Psi_m$ in the silenced cells (ΔpTPP decrease of 0.28 and 0.05 in the control and silenced cells, respectively), but the decrease was fully reversed by oligomycin, much the same as in control cells. Membrane potential measurements thus further supported the conclusion that the *ATP5E*-silenced cells are well coupled but the low content of ATP synthase complex limits the function of mitochondrial oxidative phosphorylation.

3.3. Silencing of the ATP5E gene leads to relative accumulation of F_o subunit c

An alteration of ATP synthase assembly due to low availability of ε subunit may lead to an accumulation of incomplete assemblies consisting of some enzyme subunits, e.g. F_1 ATPase subcomplexes, assuming that the ε subunit is added at the late stage of F_1 formation, i.e. after the γ and/or δ subunits. Having determined the cellular content of individual ATP synthase subunits, we found that *ATP5E* silencing reduced the content of the F_1 subunits α and ε as well as of the F_o subunits a and d (Fig. 3A) to a similar extent. The only subunit that was not reduced was the F_o subunit c. Normal content of subunit c was maintained in all cell lines with silenced *ATP5E*, demonstrating

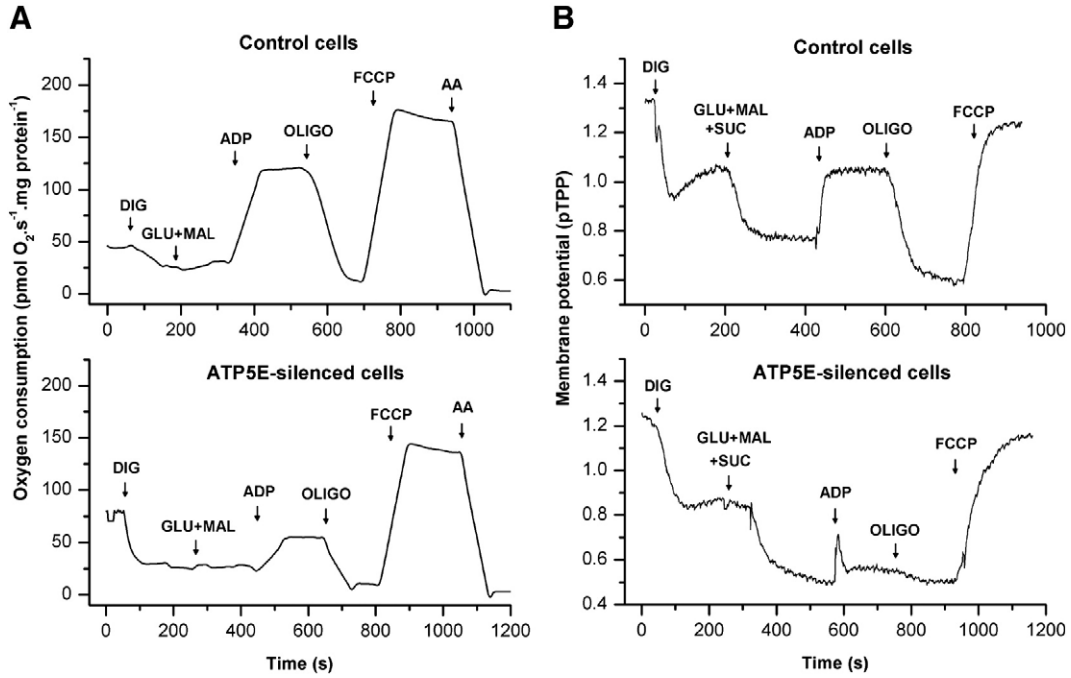

Fig. 2. ADP-stimulated respiration and ADP-induced decrease of mitochondrial membrane potential $\Delta\Psi_m$ in *ATP5E*-silenced cells. (A) Respiration and (B) $\Delta\Psi_m$ were measured in shE2a and NS cells permeabilized with digitonin (DIG) using 10 mM glutamate (GLU), 3 mM malate (MAL), 10 mM succinate (SUC), 1.5 mM ADP, 1 μM oligomycin (OLIGO), 1 μM FCCP and 1 μM antimycin A (AA). Respiration was expressed as oxygen consumption in pmol O2 s^{-1} mg $protein^{-1}$, mitochondrial membrane potential $\Delta\Psi_m$ measured with TPP^+-selective electrode was plotted as pTPP, *i.e.* natural logarithm of TPP^+ concentration (μM).

that if the ATP synthase assembly process was inhibited, the "excess" subunit c was not degraded and cleared out as other ATP synthase subunits.

To characterize further the accumulated subunit c, mitochondrial proteins were solubilized with DDM and analyzed for the content of ATP synthase subunits. As shown in Fig. 3B, the subunit c of *ATP5E*-silenced mitochondria was recovered in both soluble and insoluble fractions. In comparison with the control NS cells, both fractions from *ATP5E*-silenced cells showed a much higher content of subunit c relative to subunits α or d. Thus the solubilized proteins from *ATP5E*-silenced mitochondria were 2–2.5-fold enriched in subunit c and the DDM-insoluble pellet was enriched 4–10-fold.

When the solubilized mitochondrial proteins were subjected to 2D electrophoresis, BN/SDS-PAGE and WB analysis (Fig. 4), in control mitochondria all subunit c as well as subunit a were present in assembled F_oF_I ATP synthase complex and neither subunit c nor subunit a could be detected around 370 kDa where the F_1 subcomplex migrates. In mitochondria from the *ATP5E*-silenced cells, the subunit c signal was also present in the F_oF_1 complex, in a smaller amount, in a good correspondence with the reduced content of ATP synthase. In addition, significant signal of subunit c was found in the BN-PAGE region of about 200–400 kDa. In this region, however, no F_1 subunits α and β or F_o subunits a and d could be detected (Fig. 4).

4. Discussion

The present study demonstrates that the ε subunit is essential for the biosynthesis of F_1 catalytic part of mammalian ATP synthase complex and that a decreased amount of available subunit ε due to *ATP5E* silencing limits the cellular content of assembled and functional ATP synthase. As revealed by respiration and mitochondrial membrane potential $\Delta\Psi_m$ measurements, *ATP5E* silencing consequently decreases the activity of mitochondrial oxidative phosphorylation, not affecting the tight coupling of mitochondria. These data clearly indicate that the ε subunit plays an important role in the assembly and/or stability of F_1 moiety of mammalian ATP synthase.

The biogenesis of eukaryotic ATP synthase is a highly organized process depending on mutual action of different ancillary factors. At least 13 ATP synthase-specific factors exist in yeast. They are involved in transcription and translation of mtDNA-encoded subunits and in the assembly of the ATP synthase complex [26–29]. Much less is known about the mammalian enzyme where only 4 specific factors have been found so far. ATP11 and ATP12 are essential for the assembly of F_1 subunits α and β, similarly as their yeast homologues [30]. There is also a mammalian homologue of ATP23, yeast metalloprotease and chaperone of subunit 6 [27,31], but its function is not known. Recently, TMEM70 was identified as a novel factor of ATP synthase biogenesis in higher eukaryotes [32]. Its deficiency results in diminished amount of the full-size ATP synthase complex with detectable traces of the free F_1-part in some patients' tissues [33].

The biosynthesis and assembly of the F_1 catalytic part begins with the formation of $\alpha_3\beta_3$ oligomer catalyzed by ATP11 and ATP12 assembly factors [26] to which are then added subunits γ, δ and ε. It is not clear when and how exactly the subunit ε is inserted, but an ε null mutant of *S. cerevisiae* [17] indicated the presence of F_1 subcomplexes lacking also γ and δ subunits. Their expected size would be below that of $\alpha_3\beta_3\gamma\delta\varepsilon$ complex (ca. 370 kDa), and if they accumulate, they should be resolved by electrophoresis at native conditions. In our experiments with mammalian HEK293 cells, apparently neither ε-less F_1 molecules nor any smaller α/β-containing subassemblies could be detected after *ATP5E* silencing, indicating that either the lack of ε prevents their formation or such incomplete assemblies are very unstable and short-lived. This is in accordance with the observed low stability of F_1 in the ε null mutant in *S. cerevisiae* [17].

Another important finding of our study is that of unchanged content of subunit c in mitochondria upon *ATP5E* silencing. It demonstrates that in contrast to other ATP synthase subunits, the "excess" subunit c is not degraded. A major part of accumulated subunit c was resistant to solubilization with mild detergent DDM and likely represents insoluble and strongly hydrophobic subunit c aggregates. However, even the DDM-soluble fraction of *ATP5E*-silenced mitochondria was enriched in subunit c, this was resolved

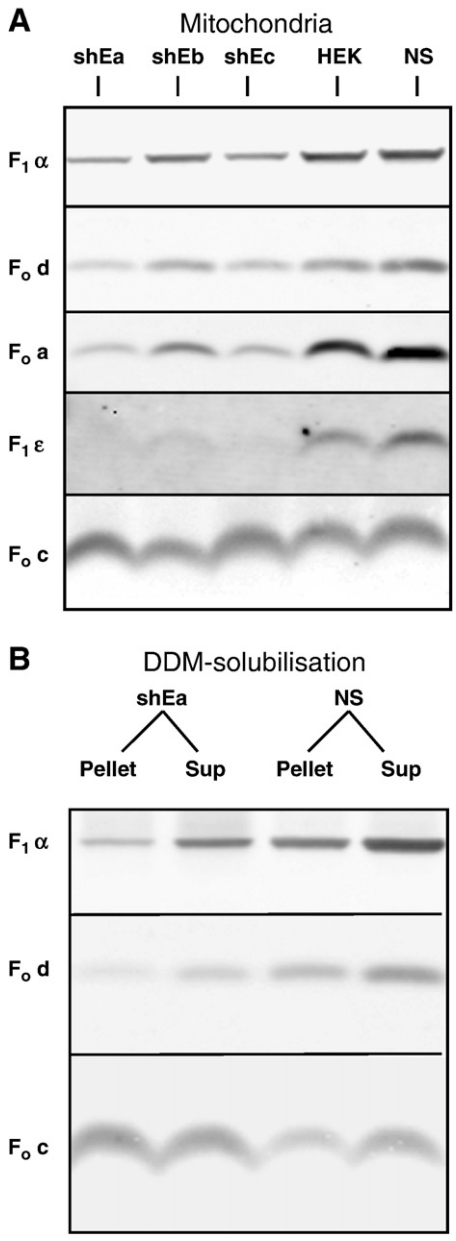

Fig. 3. Mitochondrial content of F_1 and F_o subunits in *ATP5E*-silenced cells. (A) Isolated mitochondria (10 μg protein aliquots) from silenced (shE2a, shE2b, shE2c) and control (HEK293, NS) cells and (B) 10 μg protein aliquots of 26000 ×*g* supernatant (Sup) and pellet (Pellet) from DDM-solubilized (2 g/g protein) mitochondria from silenced (shE2a) and control (NS) cells were analyzed by SDS-PAGE and WB. For detection antibodies to F_1 subunits α, and ε and F_o subunits a, d and c were used as indicated.

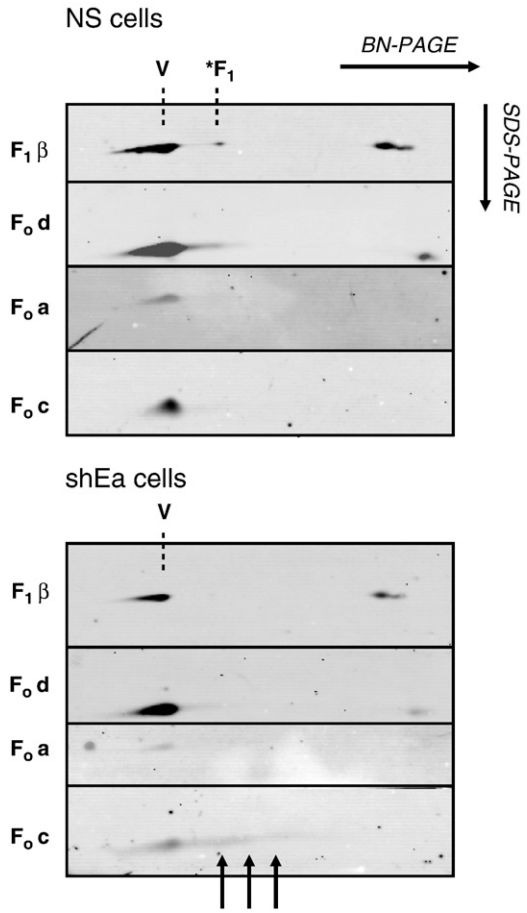

Fig. 4. Two-dimensional electrophoretic analysis of ATP synthase subunits in *ATP5E*-silenced cells. DDM-solubilized (2 g/g protein) proteins of mitochondria from shEa and NS cells were subjected to 2D electrophoresis and WB analysis was performed with indicated antibodies to ATP synthase subunits. V and $*F_1$ indicate position of ATP synthase monomer and F_1 subcomplex, arrows indicate accumulated subunit c.

by BN/SDS-PAGE in the second dimension corresponding to native complexes of about 200–400 kDa, which contained no F_1 subunits. Their origin is unclear at present, and they could represent breakdown products of subunit c oligomer attached to an unstable ε-lacking F_1 intermediate. However, their size is much larger than that of an oligomer of 10–12 copies of subunit c. Accumulated subunit c aggregates were also free of other F_o subunits, notably the subunit a, which is closely apposed to the c oligomer forming together the proton channel in the F_o structure. Apparently *ATP5E* silencing did not lead to enhanced proton conductivity of the inner mitochondrial membrane although a high proton leak was associated with ungated F_o structures in yeast ε null mutants [17]. Contrary to these findings, mitochondria of *ATP5E*-silenced cells showed an even tighter coupling than controls, in our work.

The accumulation of F_1 and larger assembly intermediates containing subunit c complexes of F_1 with subunit c oligomer were observed in various types of mammalian cultured cells or tissue samples with altered biogenesis of ATP synthase. They most likely represent dead-end products of a stalled assembly process resulting from a lack of mtDNA-encoded subunit a [34–36] or mutations in this subunit [36–38]. Upon *ATP5E* silencing in a human cell line, however, such intermediates are not present (Fig. 4). Interestingly, yeast F_1 mutants have been recently shown to inhibit translation of *ATP6* and *ATP8* mRNAs, but also in this case no F_1 or F_1-subunit c intermediates could be found in the *ΔATP15* strain [39].

Further studies are needed to resolve the properties and mechanism of subunit c accumulation, which is specifically induced by the absence of subunit ε and suggests a direct interaction and a regulatory role of ε in the assembly of ATP synthase rotor structure. Interestingly, no similar storage of subunit c could be found in mitochondria with ATP synthase deficiency of nuclear genetic origin due to mutations in *ATP12* [40] or *TMEM70* genes [32,41].

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References

[1] J.E. Walker, I.M. Fearnley, N.J. Gay, B.W. Gibson, F.D. Northrop, S.J. Powell, M.J. Runswick, M. Saraste, V.L. Tybulewicz, Primary structure and subunit stoichiometry of F1-ATPase from bovine mitochondria, J. Mol. Biol. 184 (1985) 677–701.

[2] I.R. Collinson, J.M. Skehel, I.M. Fearnley, M.J. Runswick, J.E. Walker, The F1F0-ATPase complex from bovine heart mitochondria: the molar ratio of the subunits in the stalk region linking the F1 and F0 domains, Biochemistry 35 (1996) 12640–12646.

[3] S. Anderson, A.T. Bankier, B.G. Barrell, M.H.L. de Bruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, P.H. Schreier, A.J.H. Smith, R. Staden, I.G. Young, Sequence and organization of the human mitochondrial genome, Nature 290 (1981) 457–465.

[4] S. Karrasch, J.E. Walker, Novel features in the structure of bovine ATP synthase, J. Mol. Biol. 290 (1999) 379–384.

[5] C. Gibbons, M.G. Montgomery, A.G. Leslie, J.E. Walker, The structure of the central stalk in bovine F(1)-ATPase at 2.4 A resolution, Nat. Struct. Biol. 7 (2000) 1055–1061.

[6] J.L. Rubinstein, J.E. Walker, R. Henderson, Structure of the mitochondrial ATP synthase by electron cryomicroscopy, EMBO J. 22 (2003) 6182–6192.

[7] J.E. Walker, V.K. Dickson, The peripheral stalk of the mitochondrial ATP synthase, Biochim. Biophys. Acta 1757 (2006) 286–296.

[8] B. Meyer, I. Wittig, E. Trifilieff, M. Karas, H. Schagger, Identification of two proteins associated with mammalian ATP synthase, Mol. Cell. Proteomics 6 (2007) 1690–1699.

[9] G.I. Belogrudov, B. Factor, is essential for ATP synthesis by mitochondria, Arch. Biochem. Biophys. 406 (2002) 271–274.

[10] O. Vinas, S.J. Powell, M.J. Runswick, V. Iacobazzi, J.E. Walker, The epsilon-subunit of ATP synthase from bovine heart mitochondria. Complementary DNA sequence, expression in bovine tissues and evidence of homologous sequences in man and rat, Biochem. J. 265 (1990) 321–326.

[11] J. Lai-Zhang, Y. Xiao, D.M. Mueller, Epistatic interactions of deletion mutants in the genes encoding the F1-ATPase in yeast Saccharomyces cerevisiae, EMBO J. 18 (1999) 58–64.

[12] J. Lai-Zhang, D.M. Mueller, Complementation of deletion mutants in the genes encoding the F1-ATPase by expression of the corresponding bovine subunits in yeast S. cerevisiae, Eur. J. Biochem. 267 (2000) 2409–2418.

[13] D. Stock, A.G. Leslie, J.E. Walker, Molecular architecture of the rotary motor in ATP synthase, Science 286 (1999) 1700–1705.

[14] F. Penin, G. Deleage, D. Gagliardi, B. Roux, D.C. Gautheron, Interaction between delta and epsilon subunits of F1-ATPase from pig heart mitochondria. Circular dichroism and intrinsic fluorescence of purified and reconstituted delta epsilon complex, Biochemistry 29 (1990) 9358–9364.

[15] G.L. Orriss, M.J. Runswick, I.R. Collinson, B. Miroux, I.M. Fearnley, J.M. Skehel, J.E. Walker, The delta- and epsilon-subunits of bovine F1-ATPase interact to form a heterodimeric subcomplex, Biochem. J. 314 (1996) 695–700.

[16] V. Kabaleeswaran, N. Puri, J.E. Walker, A.G. Leslie, D.M. Mueller, Novel features of the rotary catalytic mechanism revealed in the structure of yeast F1 ATPase, EMBO J. 25 (2006) 5433–5442.

[17] E. Guelin, J. Chevallier, M. Rigoulet, B. Guerin, J. Velours, ATP synthase of yeast mitochondria. Isolation and disruption of the ATP epsilon gene, J. Biol. Chem. 268 (1993) 161–167.

[18] X.J. Chen, Absence of F1-ATPase activity in Kluyveromyces lactis lacking the epsilon subunit, Curr. Genet. 38 (2000) 1–7.

[19] H.A. Bentlage, U. Wendel, H. Schagger, H.J. ter Laak, A.J. Janssen, J.M. Trijbels, Lethal infantile mitochondrial disease with isolated complex I deficiency in fibroblasts but with combined complex I and IV deficiencies in muscle, Neurology 47 (1996) 243–248.

[20] H. Schägger, G. von Jagow, Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form, Anal. Biochem. 199 (1991) 223–231.

[21] H. Schagger, G. von Jagow, Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, Anal. Biochem. 166 (1987) 368–379.

[22] A. Dubot, C. Godinot, V. Dumur, B. Sablonniere, T. Stojkovic, J.M. Cuisset, A. Vojtiskova, P. Pecina, P. Jesina, J. Houstek, GUG is an efficient initiation codon to translate the human mitochondrial ATP6 gene, Biochem. Biophys. Res. Commun. 313 (2004) 687–693.

[23] J. Houstek, U. Andersson, P. Tvrdik, J. Nedergaard, B. Cannon, The expression of subunit c correlates with and thus may limit the biosynthesis of the mitochondrial F0F1-ATPase in brown adipose tissue, J. Biol. Chem. 270 (1995) 7689–7694.

[24] A. Baracca, E. Amler, G. Solaini, G. Parenti Castelli, G. Lenaz, J. Houstek, Temperature-induced states of isolated F1-ATPase affect catalysis, enzyme conformation and high-affinity nucleotide binding sites, Biochim. Biophys. Acta 976 (1989) 77–84.

[25] A. Labajova, A. Vojtiskova, P. Krivakova, J. Kofranek, Z. Drahota, J. Houstek, Evaluation of mitochondrial membrane potential using a computerized device with a tetraphenylphosphonium-selective electrode, Anal. Biochem. 353 (2006) 37–42.

[26] S.H. Ackerman, A. Tzagoloff, Function, structure, and biogenesis of mitochondrial ATP synthase, Prog. Nucleic Acid Res. Mol. Biol. 80 (2005) 95–133.

[27] X. Zeng, W. Neupert, A. Tzagoloff, The metalloprotease encoded by ATP23 has a dual function in processing and assembly of subunit 6 of mitochondrial ATPase, Mol. Biol. Cell 18 (2007) 617–626.

[28] X. Zeng, A. Hourset, A. Tzagoloff, The Saccharomyces cerevisiae ATP22 gene codes for the mitochondrial ATPase subunit 6-specific translation factor, Genetics 175 (2007) 55–63.

[29] X. Zeng, M.H. Barros, T. Shulman, A. Tzagoloff, ATP25, a new nuclear gene of Saccharomyces cerevisiae required for expression and assembly of the Atp9p subunit of mitochondrial ATPase, Mol. Biol. Cell 19 (2008) 1366–1377.

[30] S.H. Ackerman, Atp11p and Atp12p are chaperones for F(1)-ATPase biogenesis in mitochondria, Biochim. Biophys. Acta 1555 (2002) 101–105.

[31] C. Osman, C. Wilmes, T. Tatsuta, T. Langer, Prohibitins interact genetically with Atp23, a novel processing peptidase and chaperone for the F1FO-ATP synthase, Mol. Biol. Cell 18 (2007) 627–635.

[32] A. Cizkova, V. Stranecky, J.A. Mayr, M. Tesarova, V. Havlickova, J. Paul, R. Ivanek, A.W. Kuss, H. Hansikova, V. Kaplanova, M. Vrbacky, H. Hartmannova, L. Noskova, T. Honzik, Z. Drahota, M. Magner, K. Hejzlarova, W. Sperl, J. Zeman, J. Houstek, S. Kmoch, TMEM70 mutations cause isolated ATP synthase deficiency and neonatal mitochondrial encephalocardiomyopathy, Nat. Genet. 40 (2008) 1288–1290.

[33] J. Houstek, S. Kmoch, J. Zeman, TMEM70 protein — a novel ancillary factor of mammalian ATP synthase, Biochim. Biophys. Acta 1787 (2009) 529–532.

[34] L.G. Nijtmans, P. Klement, J. Houstek, C. van den Bogert, Assembly of mitochondrial ATP synthase in cultured human cells: implications for mitochondrial diseases, Biochim. Biophys. Acta 1272 (1995) 190–198.

[35] P. Jesina, M. Tesarova, D. Fornuskova, A. Vojtiskova, P. Pecina, V. Kaplanova, H. Hansikova, J. Zeman, J. Houstek, Diminished synthesis of subunit a (ATP6) and altered function of ATP synthase and cytochrome c oxidase due to the mtDNA 2 bp microdeletion of TA at positions 9205 and 9206, Biochem. J. 383 (2004) 561–571.

[36] R. Carrozzo, I. Wittig, F.M. Santorelli, E. Bertini, S. Hofmann, U. Brandt, H. Schagger, Subcomplexes of human ATP synthase mark mitochondrial biosynthesis disorders, Ann. Neurol. 59 (2006) 265–275.

[37] J. Houstek, P. Klement, J. Hermanska, H. Houstkova, H. Hansikova, C. van den Bogert, J. Zeman, Altered properties of mitochondrial ATP-synthase in patients with a T —> G mutation in the ATPase 6 (subunit a) gene at position 8993 of mtDNA, Biochim. Biophys. Acta 1271 (1995) 349–357.

[38] L.G. Nijtmans, N.S. Henderson, G. Attardi, I.J. Holt, Impaired ATP synthase assembly associated with a mutation in the human ATP synthase subunit 6 gene, J. Biol. Chem. 276 (2001) 6755–6762.

[39] M. Rak, A. Tzagoloff, F1-dependent translation of mitochondrially encoded Atp6p and Atp8p subunits of yeast ATP synthase, Proc. Natl. Acad. Sci. USA 106 (2009) 18509–18514.

[40] L. De Meirleir, S. Seneca, W. Lissens, I. De Clercq, F. Eyskens, E. Gerlo, J. Smet, R. Van Coster, Respiratory chain complex V deficiency due to a mutation in the assembly gene ATP12, J. Med. Genet. 41 (2004) 120–124.

[41] J.A. Mayr, J. Paul, P. Pecina, P. Kurnik, H. Förster, U. Fötschl, W. Sperl, J. Houstek, Reduced respiratory control with ADP and changed pattern of respiratory chain enzymes due to selective deficiency of the mitochondrial ATP synthase, Pediatr. Res. 55 (2004) 1–7.