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Cellular and Functional Analysis of Four Mutations Located in the Mitochondrial *ATPase6* Gene

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ABSTRACT

The smallest rotary motor of living cells, F0F1-ATP synthase, couples proton flow—generated by the OXPHOS system—from the intermembrane space back to the matrix with the conversion of ADP to ATP. While all mutations affecting the multisubunit complexes of the OXPHOS system probably impact on the cell's output of ATP, only mutations in complex V can be considered to affect this output directly. So far, most of the F0F1-ATP synthase variations have been detected in the mitochondrial *ATPase6* gene. In this study, the four most frequent mutations in the *ATPase6* gene, namely L156R, L217R, L156P, and L217P, are studied for the first time together, both in primary cells and in cybrid clones. Arginine ("R") mutations were associated with a much more severe phenotype than Proline ("P") mutations, in terms of both biochemical activity and growth capacity. Also, a threshold effect in both "R" mutations appeared at 50% mutation load. Different mechanisms seemed to emerge for the two "R" mutations: the F1 seemed loosely bound to the membrane in the L156R mutant, whereas the L217R mutant induced low activity of complex V, possibly the result of a reduced rate of proton flow through the A6 channel. J. Cell. Biochem. 106: 878–886, 2009.

KEY WORDS: MITOCHONDRIA; F1F0-ATPase; *ATPase6* MUTATIONS; OS-ATPase; LEIGH SYNDROME

Human F0F1-ATP synthase (complex V of the respiratory chain) couples the synthesis of ATP from ADP and inorganic phosphate with the passage of protons from the intermembrane space to the matrix [Elston et al., 1998; Noji and Yoshida, 2001]. This rotary motor is comprised of at least 14 nucleus-encoded subunits (α, β, γ, δ, ε, b, c, d, e, f, g, h, IF1, and OSCP) and two mtDNA-encoded subunits (ATPase 6 and ATPase 8). The F0 portion of the complex is embedded in the mitochondrial inner membrane and contains a ring of c subunits surrounding a central γ subunit "stalk" that rotates within the F1 portion of the complex. The latter, in turn, consists of three α/β-dimers protruding into the matrix. ATPase 6, which is also part of F0, forms a channel through which proton flow is coupled with rotation of the c ring [Rastogi and Girvin, 1999; Hutcheon et al., 2001]. Several mutations have been described in the mitochondrial *ATPase6* gene. The first description of a mutation, a T > G conversion at nt-8993 (L156R), dates back to 1990 [Holt et al., 1990] and concerned a patient with the neuropathy, ataxia, retinitis pigmentosa (NARP) syndrome. Since then, variable clinical expression between families has been reported [Tatuch et al., 1992], and two main phenotypes identified: NARP and maternally inherited Leigh syndrome (MILS), distinguished by different degrees of heteroplasmy of the T8993G mutation. A different mutation, a T > C transition, at nt-8993 (L156P), has also been found in association with the different clinical presentations of NARP/MILS [de Vries et al., 1993; Santorelli et al., 1994; Fujii et al., 1998]. A similar phenotypic heterogeneity was observed for mutations at nt-9176 [i.e., T > G (L217R) and T > C (L217P)] [Thyagarajan et al., 1995; Campos et al., 1997; Dionisi-Vici et al., 1998; Carrozzo et al., 2001]. In both cases, the T > G mutation was clinically more severe than the T > C mutation. Moreover, both T > G mutations clearly impaired ATP synthesis in vitro, whereas the T > C mutations displayed a less marked effect on complex V activity [Thyagarajan

Abbreviations used: NARP, neuropathy, ataxia, and retinitis pigmentosa; MILS, maternally inherited Leigh syndrome; OXPHOS, oxidative phosphorylation; "R," either L156R or L217R mutations; "P," either L156P or L217P mutations.

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et al., 1995; Vazquez-Memije et al., 1998; Carrozzo et al., 2001]. Apart from the nucleotides T8993 and T9176, which appear to be hot-spots for mutations, few additional changes have been described in the *ATPase6* gene [De Meirleir et al., 1995; Moslemi et al., 2005]. More recently, the first mutation in the mitochondrial *ATPase8* gene (G8529A; W55X) has been described [Jonckheere et al., 2008].

In this article, we evaluated the pathogenetic mechanisms of the four most frequent mutations in the *ATPase6* gene: L156R, L217R, L156P and L217P. This is the first time that these four mutations have been analyzed simultaneously, in primary cells and in cybrid clones, across biochemical and cellular studies. A profound energy defect was found in the presence of the "R" mutation at both codons, 156 and 217. However, despite the presence of an overlapping biochemical defect, different mechanisms are suggested.

MATERIALS AND METHODS

CELL CULTURES

Human cultured skin fibroblasts were obtained from a still unreported MILS patient harboring the L156R, from a Leigh-like patient having the L156P [patient 3 in Vilarinho et al., 2001], and from the index patients reported by Dionisi-Vici et al. [1998] (mutation L217P) and by Carrozzo et al. [2001] (mutation L217R). All the mutations were virtually homoplasmic when tested in cultured cells. We also used skin fibroblast cells from age-matched controls, that is, children who underwent punch skin biopsy for dermatological diseases. Fibroblast cell lines were used to determine growth capacity, and to test the sensitivity to oligomycin during ATPase activity, in basal condition and after sonication.

FUSION PROCEDURE AND CULTURE CONDITIONS

Heteroplasmic skin fibroblasts, established from relatives of MILS patients harboring the L156R, L217R, and L156P mutations, and nearly homoplasmic skin fibroblasts harboring the L217P in the *ATPase6* gene, were used to obtain cytoplasts. The cells were enucleated and fused with a Rho0 cell line derived from 143B/TK$^-$ osteosarcoma cells according to a described methodology [King and Attardi, 1989]. Individual clones were isolated and the mutant mtDNA load was tested using appropriate PCR-restriction fragment length polymorphism (RFLP) strategies. Six clones for the L156R mutation (0%, 2%, 20%, 50%, 80%, and 100% mutant mtDNA), four for the L217R mutation (0%, 84%, 95%, and 100%), six for the L156P mutation (0%, 35%, 50%, 70%, 88%, and 100%), and the parental cell line 143B/TK$^-$ Rho+ (Rho+) were used. Only 100% mutant mtDNA clones could be obtained from the fusion of the L217P fibroblast cell line.

GROWTH CURVE

To assess the capacity to grow of primary cells, 5×10^4 fibroblasts were plated in 35-mm tissue culture dishes containing either glucose (4.5 g/L), galactose (5 mM), oligomycin (0.1 ng/ml), or galactose plus oligomycin (5 mM and 0.1 ng/ml, respectively). Fibroblasts were grown in the different media for 20 days, and cells from duplicate plates were trypsinized and counted at daily interval of 5, 10, and 20. All cell lines used had comparable passage numbers, ranging from P10 to P18 in control fibroblasts and from P7 to P15 in patients' fibroblasts.

ATP SYNTHESIS

ATP synthesis was assayed spectrophotometrically, as reported elsewhere [Garcia et al., 2000; Carrozzo et al., 2004]. Briefly, fresh mitochondria (250–350 μg protein) were incubated in 20 mM Tris–HCl, 150 mM sucrose, 1 mM ADP, 20 mM phosphate, 5 mM $MgCl_2$, 100 μM diadenosine pentaphosphate, 10 mM glucose, 30 units of hexokinase, and 50 mM succinate, (pH 7.5) at 37°C in vials with vigorous stirring to ensure maximal oxygenation. The reaction was started with 50 mM succinate and stopped after 15 min with 25 mM EDTA + 2 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP). The synthesized glucose 6-phosphate was oxidized by NADP in the presence of 30 units of glucose 6-phosphatedehydrogenase. NADPH formation was monitored at 340 nm.

ATPase ACTIVITY

ATP hydrolysis was assayed at 25°C by a spectrophotometric method in which ATP is regenerated through the action of pyruvate kinase in the presence of excess phosphoenol pyruvate [Vazquez-Memije et al., 1998]. The reaction was started by the addition of 100 μg of mitochondrial protein. Sonication was carried out at 0–4°C in an Ultrasonic Desintegrator (Vibra Cell VCX 400, Sonic & Materials Inc., Danbury, CT). Isolated mitochondria were suspended in 250 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl pH 7.5. Aliquots of 10 μl containing 100 μg of proteins were taken at different times of sonication and ATPase activity was determined in the presence or absence of oligomycin (50 μg/mg protein).

POLAROGRAPHIC STUDIES

To study oxidative phosphorylation, mitochondria were suspended at a concentration of 2.5–4.0 mg protein/ml in respiratory buffer consisting of 0.25 M mannitol, 0.2 mM EDTA, 1 mM $MgCl_2$, 10 mM KCl, 10 mM K_2HPO_4 (pH 7.2). Succinate, as oxidisable substrate, and ADP were added to a final concentration of 10 and 0.3 mM, respectively. An oxygraph fitted with a Clark electrode (type CB1D, Hansatech Instruments Ltd., Kings Lynn, England) was used to measure the respiratory rate.

Protein content for all the experiments was measured by BCA (Pierce, USA) and read at 562 nm.

STATISTICS

Statistical analysis of the data was performed using Student's *t*-test. As a general rule, only *P* values of less than 0.01 were considered significant.

RESULTS

GROWTH CURVE

The effect of galactose and oligomycin on fibroblasts growth capacity is reported in Figure 1. When galactose replaced glucose in the culture medium, the L156R mutated fibroblasts displayed a growth reduction of 49% after 5 days of incubation, and 79% after 20 days, with respect to the same cells grown in regular medium

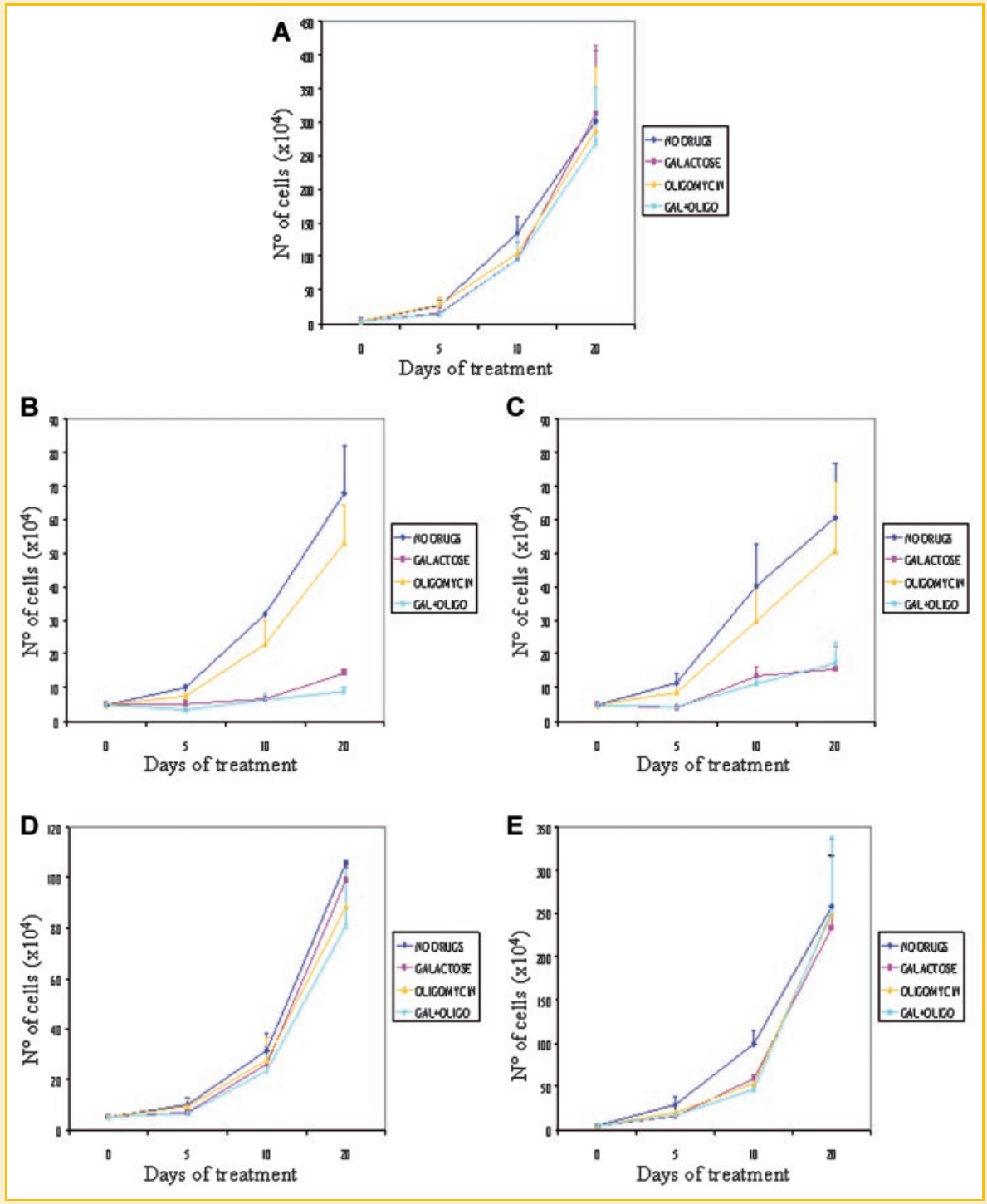

Fig. 1. Effect of galactose and oligomycin on fibroblasts growth capacity. When galactose, or galactose plus oligomycin were added to the culture medium, the two "R" mutations showed a much more severe phenotype than the "P" mutations. The addition of 0.1 ng/ml oligomycin alone produced a not significant effect in all cell lines tested. A: Control; (B), L156R; (C), L217R; (D), L156P; (E), L217P. All experiments were performed in 100% mutated fibroblasts. The results are expressed as mean values ± SE of at least three experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 1B). In the L217R cell line (Fig. 1C) the growth capacity was reduced by 65% at 5 days, and 75% at 20 days. The two "P" mutated fibroblasts (Fig. 1D–E) displayed a behavior similar to that of control cell lines (Fig. 1A). The addition of 0.1 ng/ml of oligomycin produced a not significant effect in all cell lines tested, including the control. Consistently, the simultaneous addition of galactose and oligomycin reproduced the effect of the galactose treatment (Fig. 1A–E).

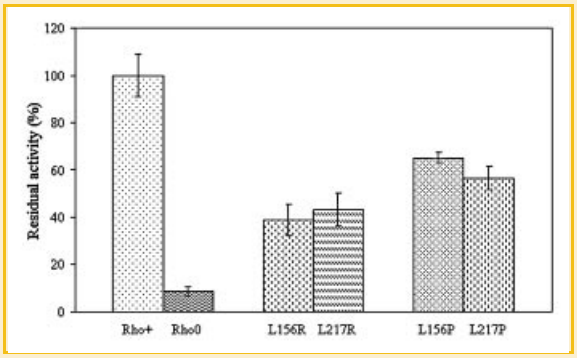

Fig. 2. ATP synthesis in 100% mutated cybrid clones. ATP production was significantly ($P < 0.001$) reduced in all clones when these were compared with Rho+ control cell lines. Furthermore, a similar statistical difference ($P < 0.001$) in ATP production was found between the "R" and "P" mutations at codon 156. A $P < 0.01$ value was observed for the mutations at codon 217. The results are expressed as % of residual activity ± SD of at least 10 experiments.

ATP SYNTHESIS IN 100% MUTATED CLONES

Figure 2 shows the percentage of residual activity of the ATP synthesis in the 100% mutated clones of the four mutations herein described. The activity amounted to 22, 24, 37, and 32 nmol ATP/min/mg protein in the L156R, L217R, L156P, and L217P mutated clones, respectively. The activity of the Rho+ control cell line was 57 nmol ATP/min/mg protein. The two "R" clones showed a reduction of about 60% in ATP production compared to Rho+, whereas a lower inhibition of ATP synthesis—around 40%—was observed in the two "P" clones. Unsurprisingly, ATP synthesis in Rho0 cells was severely impaired.

The activity in mutant and control cells was also measured in the presence of oligomycin (10 μg/mg protein), the specific inhibitor of complex V. As expected in this condition, ATP synthesis was reduced by more than 90% (data not shown).

ATP SYNTHESIS IN CLONES WITH DIFFERENT MUTATION LOAD

In order to study the effects of the different mutations on complex V, and also to estimate their threshold effects, mitochondria isolated from cybrid clones containing different mutant load were assayed for their ability to synthesize ATP (Fig. 3). With the L156R mutation, the reduced rate of ATP production for each strain correlated well with the percentage of mutated DNA present in the mitochondria when expressed as percentage of the wild-type (0% mutated). Mitochondria containing 100% mutated DNA showed a rate of ATP synthesis corresponding to only 25% of that displayed by the wild-type (Fig. 3A). Moreover, the activity of complex V was significantly altered ($P < 0.01$) starting from the clone having 50% mutant mtDNA genomes. Similarly, the L217R mutated clones showed a rate of ATP production that decreased as the percentage of mutated DNA increased. The 100% mutated clone displayed a rate of ATP synthesis corresponding to 49% of that shown by its corresponding wild type clone (Fig. 3B). On the other hand, the level of ATP production was similar in all the clones harboring the L156P mutation, regardless of the mutant load. In particular, 100% mutated clones did not significantly differ from wild-type ones (Fig. 3C).

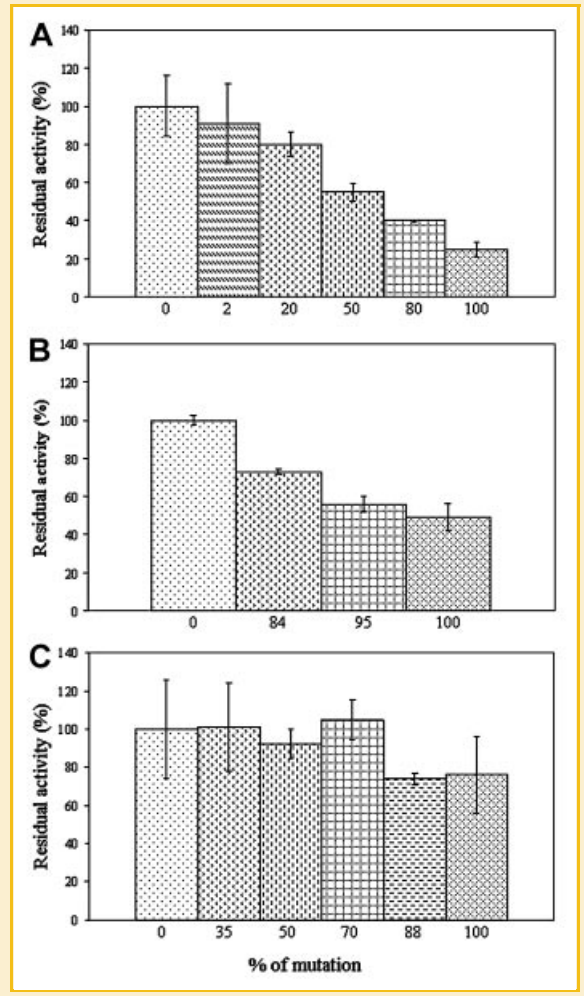

Fig. 3. ATP synthesis in cybrid cell lines with different mutation load. The L156R (A) and L217R (B) mutated clones showed an ATP production that decreased as the percentage of mutated DNA increased. This reduction reached significance ($P < 0.01$) at 50% mutation load with the L156R mutation and at 84% mutation load with the L217R mutation. With the L156P mutation, not significant difference in the level of ATP synthesis in all clones was observed (C). The results are expressed as % of residual activity ± SD of at least three experiments.

EFFECT OF OLIGOMYCIN AND SONICATION ON ATPase ACTIVITY

It is well known that oligomycin inhibits ATP synthesis and hydrolysis by different mechanisms. During synthesis, F1 should be bound to the membrane in order to be inhibited by oligomycin which binds to the ATPase 6 subunit blocking proton translocation. Isolated F1 does not make ATP, it is not sensitive to oligomycin, and shows a higher ATP hydrolysis than the F1F0 complex. On the other hand, it is generally agreed that mechanical treatment of mitochondria, such as sonication, can release F1-ATPase from the membrane. In this study, the effect of oligomycin or conditions altering the sensitivity to oligomycin—like sonication—were analyzed to determine their implications for ATPase 6 mutated subunit and complex V functioning. Figure 4A shows the effect of oligomycin (50 μg/mg protein) on the ATPase activity in mitochondria isolated from virtually homoplasmic fibroblasts, and

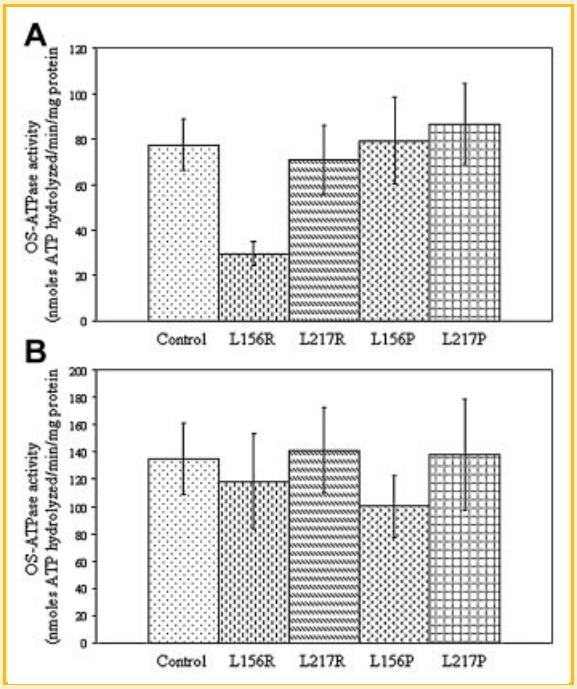

Fig. 4. Effect of oligomycin on ATPase activity in fibroblasts (panel A) and in cybrid clones (panel B). Mitochondria freshly isolated from normal and mutated cells were assayed for ATP hydrolysis in the presence of 50 μg oligomycin/mg protein. The L156R mutated fibroblasts showed low OS-ATPase, with a reduction of 62% ($P = 0.001$). In cybrid clones the OS-ATPase activity was similar to the control cell lines. The results are expressed as mean values ± SD of at least four experiments.

it was expressed as oligomycin sensitive ATPase (OS-ATPase). Whilst cell lines containing the L217R, or L156P, or L217P mutations showed an OS-ATPase activity similar to that of controls, cells harboring the L156R mutation showed a reduction of 62%. These effects could be replicated in the 100% mutant cybrids, except that 100% mutant L156R clones showed a not significant ATPase activity reduction (Fig. 4B).

Figure 5 illustrates the effect of sonication on the enzyme's sensitivity to oligomycin, in virtually homoplasmic cultured fibroblasts and 100%-mutated cybrid clones. The mitochondria obtained from control cells showed no significant changes in sensitivity to oligomycin during sonication (Fig. 5A). Mutations at residue 217 (Fig. 5C,E) and the L156P variant (Fig. 5D) did not affect the sensitivity to oligomycin during sonication in either cell lines used, displaying a behavior similar to that of the controls. Contrariwise, the L156R mutation (Fig. 5B) displayed a rapid and significant loss of the sensitivity to oligomycin in cultured skin fibroblasts (reduction ranging from 24% to 47%), no such effect was observed in 100% mutant cybrids. These results are consistent with data presented in Figure 4A,B.

POLAROGRAPHIC STUDIES

Polarographic measurements of the overall oxidative phosphorylation activity provide a more general assessment of mitochondrial metabolism [Millis and Pious, 1973]. Compared to their own controls cell lines (clone 0% mutated) both 100% mutated "R" clones showed a reduction by 33% of state 3, less respiratory control index and an ATP synthase activity decreased by 50% (Table I). Conversely, the L156P mutation did not affect the rate of state 3, the respiratory control index and the ATP synthase activity calculated from state 3. It was not possible to compare the L217P mutation to its own control, but this clone showed a behavior not very far from that of the other "P" mutated clone and Rho+ cell line.

DISCUSSION

The pathogenic consequences of the NARP/MILS associated mutations in the *ATPase6* gene—L156R, L217R, L156P, and L217P—have been studied largely in primary cells [Tatuch and Robinson, 1993; Thyagarajan et al., 1995; Vazquez-Memije et al., 1998; Carrozzo et al., 2001] and transmitochondrial cybrids [Trounce et al., 1994; Manfredi et al., 1999] but never simultaneously to pinpoint specific differences which might be relevant to the severity of the associated phenotypes. To expand our understanding of the pathogenesis, and disclose possible similarities or differences, we evaluated for the first time together the effects of the L156R, L217R, L156P, and L217P mutations in primary cells and in cybrid clones, across biochemical and cellular studies. Since nuclear genetic factors are known to influence the expression of mtDNA variants, we established an osteosarcoma transmitochondrial cell system to analyze the four mutations and to generate different mutation loads. Since all the cybrids came from the same source we were able to compare products of genomes that were identical but for the mutations under study.

Our analyses showed that, in regular medium, all mutant fibroblasts displayed a reduced growth capacity when compared to normal cells, being with "R" mutations more markedly reduced than "P." This impaired phenotype was even more marked when galactose was used instead of glucose, in agreement with the notion that fibroblasts from patients with different mitochondrial disorders grow poorly when galactose is the only carbon source [Robinson et al., 1992; Robinson, 1996]. On the other side, it has been shown [Manfredi et al., 1999] that fibroblasts harboring high levels of the L156R mutation have decreased ATP synthesis activity, but do not display any growth defect under standard culture conditions as well as in presence of galactose. This apparent incongruity between our present findings and works done by others can be explained by the longer incubation time needed to make the phenomenon becoming evident. On the other hand, when oligomycin and galactose were used together, the reduced growth capacity of the "R" and "P" fibroblasts reflected mostly the effect of galactose, which had been underestimated in the previous study. Thus, it seems that the two "R" mutations are more glycolytic than the "P" mutations.

In agreement with previous data in primary cells [Tatuch and Robinson, 1993; Thyagarajan et al., 1995; Santorelli et al., 1996; Vazquez-Memije et al., 1998; Carrozzo et al., 2001], we observed that ATP synthesis in all the 100% mutated clones was more affected when leucine is substituted by arginine. In fact, "R" mutated clones showed a similar and considerable inhibition of ATP production,

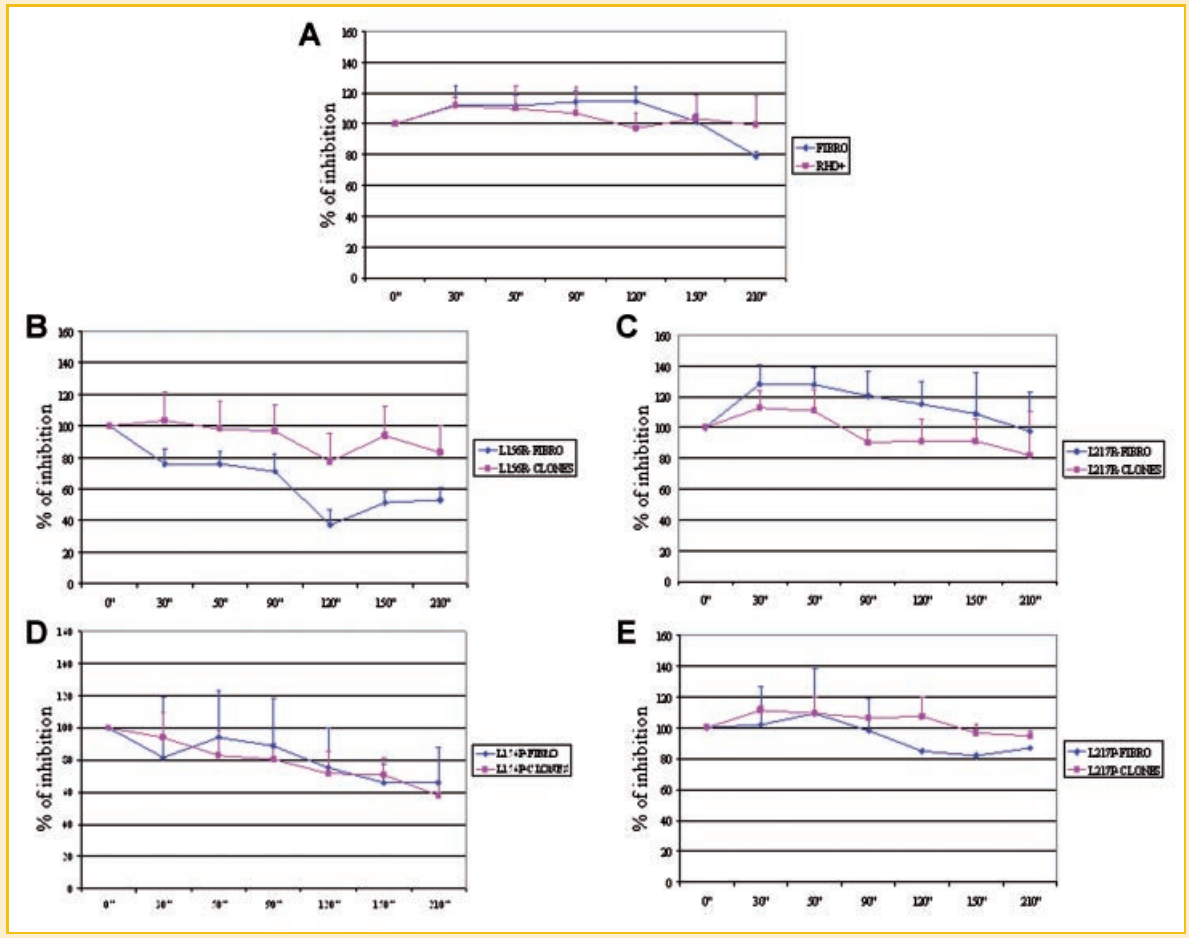

Fig. 5. Effect of sonication on oligomycin-sensitive ATPase in fibroblasts (blue lines) and 100% mutated cybrids (pink lines). ATPase activity and sensitivity to oligomycin during sonication of mitochondria isolated from control cell lines (panel A), L217R (panel C), L217P (panel E), and L156P (panel D) mutated cells, did not show any variation. Contrariwise, the L156R mutation (panel B) in cultured fibroblasts, generates a rapid and significant loss of sensitivity to oligomycin, starting at 20″ of sonication, no such effect was observed in 100% mutant cybrids. In the X-axis is reported the different time (seconds) of sonication. The results are expressed as mean values ± SD of at least four determinations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

whereas a lower inhibition of ATP synthesis was detected in the "P" mutated clones. Examination of oxidative phosphorylation capacity in cybrid clones also confirmed the deleterious effect of the "R" mutations, and could reflect well the different clinical severity of patients harboring the different mutations [Schon et al., 2001]. Consistently, the progressive raise in mutant load correlates well with the decreased ability of isolated mitochondria to synthesize ATP, at least for the "R" clones, and mirror the gradient of clinical phenotypes observed in affected patients even within a family [Tatuch et al., 1992, 1994; Carrozzo et al., 2001]. In contrast, no correlation between ATP synthesis and different mutation loads was observed in the L156P clones. The biochemical results, reported here, correlate well with the relatively milder clinical manifestations already described [de Vries et al., 1993; Santorelli et al., 1994, 1996; Fujii et al., 1998]. On the whole, reduced growth capacity, strong sensitivity to metabolic stressors and lower production of ATP are peculiar features of the "R" mutations, which depend strongly upon the level of mutated mtDNA genomes, and correlate well with severity of clinical features.

It is renowned that the F1F0-ATPase is characterized by two activities which can be separated physically and functionally into a proton channel (F0) and a soluble catalytic domain (F1). ATP synthesis can occur only when these two parts are tightly coupled. F1 can be readily removed from the membrane, and in the soluble form it functions essentially as an ATP hydrolase. Upon removal of F1, the membrane fraction works principally as a passive proton channel and the ATPase activity of soluble F1 is insensitive to oligomycin, which is known to bind to the F0-subunit [Breen et al., 1986]. Our data strongly suggest that in primary cells the L156R mutation exhibits a catalytic F1 less tightly bound to the membrane, since the hydrolytic activity was not fully sensitive to oligomycin, and 20 sonications' seconds resulted in loss of its sensitivity to the inhibitor. This is in agreement with previous observations in EDTA-submitochondrial particles where inhibition by oligomycin of ATPase activity in cells harboring the L156R mutation was 20% less than in controls [Vazquez-Memije et al., 1998; Cortes-Hernandez et al., 2007], and with a more "labile" enzyme in situ as suggested [Sgarbi et al., 2006]. The positive charge introduced by the arginine

TABLE I. Oxidative Phosphorylation in Mitochondria Isolated From Clones

Clone	State 3[a]	State 4[a]	Respiratory control index[b]	ADP/O[c]	ATP synthase[d]
T8993G 0% mutant	13.67 ± 1.20	5.47 ± 0.54	2.49 ± 0.30	2.13 ± 0.28	60.0 ± 9.0
100% mutant	9.39 ± 0.20	5.39 ± 0.34	1.74 ± 0.09	1.82 ± 0.35	31.29 ± 5.54
T8993C 0% mutant	27.49 ± 1.38	12.37 ± 0.86	2.22 ± 0.19	2.20 ± 0.15	86.0 ± 7.7
100% mutant	25.56 ± 1.55	13.20 ± 1.52	1.93 ± 0.15	2.40 ± 0.22	78.5 ± 6.8
T9176G 0% mutant	15.78 ± 1.31	6.70 ± 1.17	2.40 ± 0.43	2.13 ± 0.13	61.46 ± 7.62
100% mutant	10.33 ± 0.38	5.66 ± 0.52	1.83 ± 0.24	1.91 ± 0.40	30.14 ± 4.68
T9176C 0% mutant	n.d.	n.d.	n.d.	n.d.	n.d.
100% mutant	24.70 ± 4.79	9.46 ± 1.68	2.61 ± 0.30	1.45 ± 0.24	54.80 ± 7.77
Rho+	16.80 ± 1.75	6. 94 ± 1.14	2.44 ± 0.42	2.23 ± 0.45	70.43 ± 12.72

[a]nmol O_2 consumed/min/mg protein.
[b]Respiratory control index: state 3/state 4.
[c]ADP/O ratio: ADP molecules phosphorylated to atoms of oxygen consumed.
[d]ATP synthase activity was calculated from the duration of state 3 and expressed as nmol ADP added (150 nmol) per min per mg protein.

at residue 156 in a transmembrane helix could reduce the thermodynamic stability of the protein in the membrane and disturb the interaction between the enzyme and the membrane. This issue is still controversial, since impaired F1F0 assembly, both in human as well as in *E. coli* [Hartzog and Cain, 1993; Houstek et al., 1995; Carrozzo et al., 2000; Nijtmans et al., 2001], enzyme uncoupling [Sgarbi et al., 2006], and proton conduction blockade through F0 [Garcia et al., 2000; Cortes-Hernandez et al., 2007], have all been hypothesized. The not evident effects on the F0–F1 bond observed in 100% cybrids might reflect the different cell type, where the different membrane lipid milieu of tumoral cells versus fibroblasts playing a regulatory role in determining the activities of mitochondrial membrane bound enzymes [Daum, 1985] is one hypothesis.

To summarize, our biochemical and growth rate analyses showed that (i) the "R" mutations have a more severe phenotype than the "P" mutations, (ii) there is a threshold effect in both "R" changes, and that (iii) the amino acid change at residue 156 seems to be more severe than residue 217. Also, the L156R mutation appears to produce an F1 portion that is loosely bound to the membrane considering the low OS-ATPase activity, whereas the L217R mutant induces low activity of complex V, possibly reducing the rate of proton flow through the A6 channel. The two "P" mutations, although less severe, are still able to affect ATP production and growth rate. These findings can be tentatively explained in the face of the proposed *a–c* coupling model of F1F0–ATP synthase [Rastogi and Girvin, 1999] attempting some insight into the pathomechanisms of mitochondrial *ATPase6* mutations [Schon et al., 2001; Sgarbi et al., 2006]. Following this model, residues R159 (in the *a* subunit) and E58 (in the *c* subunit), and their interactions, appear crucial for proton flow, movement of the c-ring, conformational change of α–β subunits, and finally for the synthesis of ATP. Our findings suggest that some differences, site-related, should be considered between the two "R" mutations. The amino acid L156 makes a direct *a–c* inter-helix contact with A50 and lies close to residue R159, which also makes an important *a* inter-helix contact with Q210. Instead, residue L217 is close to Q210 and interacts only indirectly with R159 (Fig. 6). The replacement of a highly conserved neutral amino acid (leucine) with a basic amino acid (arginine) at either site "slows down" the efficiency of ATP synthesis, by impairing proton flow through the F0 portion of the enzyme. However, the close vicinity of the R156 mutated amino acid to the R159 residue possibly weakens the connections of E58 and Q210 to R159 and produces a more severe phenotype due to inappropriate interaction, followed by reduced rotary coupling,

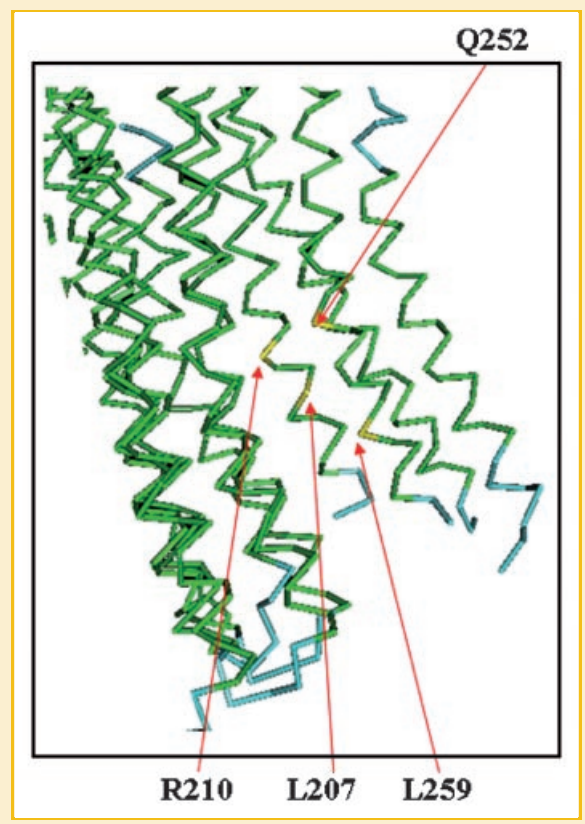

Fig. 6. *Escherichia coli* structural model of F1F0 ATP synthase. A structural modeling of the A1c12 Subcomplex of F1F0 ATP synthase. The model was drawn with CnD3 4.1 ("http://www.ncbi" www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml) based on the ATPase subunit a [MMDB: 11491; PDB: 1C17] as template. Zooming on the amino acid residues L207 (=human L156), R210 (=human R159), Q252 (=human Q210), L259 (=human L217) is shown with an arrow. It should be noticed that PDB file 1C17 contains 13 polypeptide chains, 12 of which are individual c subunits, the last being four helices of the *a* subunit. Only the representation of each individual subunit *c* is based on atomic structure data, while the c-ring, and subunit *a*, is merely predicted on the computational modeling based on Rastogi and Girvin [1999]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

between *a* and *c*. Mutations replacing leucine for proline are less simply understandable but it is tempting to imagine that the mutant enzyme might result in a distorted, yet partially functional, proton channel and hinder in some way either proton flow through the F0 portion or A6–*c* contact, or both. Under this hypothesis, which requires further investigation, the replacement at codon 156 produces a greater damage than that at codon 217, as shown by adopting an in silico model of ATPase *a* subunit (http://bp.nuap.nagoya-u.ac.jp/sosui; www.ch.embnet.org/cgi-bin/TMPRED; www.cbs.dtu.dk/services/TMHMM-2.0).

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