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Replacement of the C6ORF66 Assembly Factor (NDUFAF4) Restores Complex I Activity in Patient Cells

Dana Marcus,[1] Michal Lichtenstein,[1] Ann Saada,[2] and Haya Lorberboum-Galski[1]

[1]Department of Biochemistry and Molecular Biology, Institute for Medical Research Israel-Canada (IMRIC), Hebrew University–Hadassah Medical School, Jerusalem, Israel; and [2]Monique and Jacques Roboh Department of Genetic Research, Department of Genetics and Metabolic Diseases, Hadassah, Hebrew University Medical Center, Jerusalem, Israel

Disorders of the oxidative phosphorylation (OXPHOS) system frequently result in a severe multisystem disease with the consequence of early childhood death. Among these disorders, isolated complex I deficiency is the most frequently diagnosed, accounting for one-third of all cases of respiratory chain deficiency. We chose to focus on complex I deficiency, caused by mutation in the assembly factor chromosome 6, open reading frame 66 (C6ORF66; NADH dehydrogenase (ubiquinone) complex I assembly factor 4 (NDUFAF4)) protein. We used the approach of cell- and organelle-directed protein/enzyme replacement therapy, with the transactivator of transcription (TAT) peptide as the moiety delivery system. This step will enable us to deliver the wild-type assembly factor C6ORF66 into patient cells and their mitochondria, leading to the proper assembly and function of complex I and, as a result, to a functional OXPHOS system. We designed and constructed the TAT-ORF fusion protein by gene fusion techniques, expressed the protein in an *Escherichia coli* expression system and highly purified it. Our results indicate that TAT-ORF enters patients' cells and their mitochondria rapidly and efficiently. TAT-ORF is biologically active and led to an increase in complex I activity. TAT-ORF also increased the number of patient cells and improved the activity of their mitochondria. Moreover, we observed an increase in ATP production, a decrease in the content of mitochondria and a decrease in the level of reactive oxygen species. Our results suggest that this approach of protein replacement therapy for the treatment of mitochondrial disorders is a promising one.

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INTRODUCTION

A large number of mutations in either the mitochondrial DNA (mtDNA) or the nuclear genome (1) cause mitochondrial diseases. Among the defects in the oxidative phosphorylation (OXPHOS) system, deficiency of the respiratory chain complex I (NADH CoQ oxidoreductase, EC 1.6.5.3) is the most common, accounting for one-third of all patients referred for OXPHOS evaluation (2). Complex I is the first complex of the mitochondrial respiratory chain catalyzing the transfer of electrons from NADH to coenzyme Q, while shuttling protons through the mitochondrial inner membrane. This large intricate complex is composed of 44 structural subunits, seven of which are encoded by the mtDNA, whereas 37 are nuclear encoded (3).

During the past two decades, only 33% of the complex I deficiencies have been explained by mutations in these genes (4). To explain most of the remaining cases, it was assumed that complex I assembly factors must exist analogous to the 20 or more factors found essential for complex IV assembly. Indeed, in 2002, the first complex I assembly factor was discovered (5), and, in 2005, one of these factors was implicated for the first time in complex I deficiency (6). Since then, genetic, bioinformatic and proteomic analyses have led to the identification of no less than 10 assembly factors (7). A number of complex I assembly factors are linked to human diseases (4,8–14); among them is the chromosome 6, open reading frame 66 (C6ORF66) assembly factor (15).

The *C6orf66* gene product, termed hormone-regulated proliferation associated protein (HRPA20), was first identified as a prooncogene and was demonstrated to promote breast cell cancer invasiveness by inducing the excretion of the extracellular-matrix-degrading enzyme matrix metallopeptidase 9 (MMP-9) (16). In 2008, the same protein was identified by Saada and her coworkers (15), in collaboration with our laboratory as an assembly factor.

Using C6ORF66 antibodies, it was demonstrated that the C6ORF66 protein is localized within the mitochondria. The first 34 residues of the protein are pre-

Address correspondence to *Haya Lorberboum-Galski, Department of Biochemistry and Molecular Biology, Institute for Medical Research Israel-Canada, Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel. Phone: +972-2-6757465; Fax: +972-2-64158485; E-mail: hayag@ekmd.huji.ac.il.*
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dicted to form the mitochondrial-targeting sequence (MTS). The exact role of C6ORF66—later termed "NDUFAF4" (NADH dehydrogenase [ubiquinone] complex I assembly factor 4)—in complex I biogenesis is not yet fully understood; however, it was reported that together with the assembly factor NDUFAF3, these two proteins participate at early stages of complex I assembly (9). Pathogenic mutations in the gene encoding C6ORF66 result in fatal neonatal mitochondrial disease with severe complex I enzymatic deficiency (15). Isolated muscle mitochondria from a patient with a homozygous mutation retain only 30% residual mature complex I, with the accumulation of stalled assembly intermediates (15).

Four intervention strategies for mitochondrial dysfunction have been described, including genetic therapy, the use of small molecules, metabolic manipulation, diet and exercise (1). However, most cases are not fully treatable. Thus, physicians are desperately seeking new therapeutic options to deal with these types of disorders.

Enzyme replacement therapy is a relatively new approach to the treatment of metabolic disorders whereby the deficient or absent enzyme is artificially manufactured, purified and given to the patient on a regular basis. Enzyme replacement therapy has been successfully accepted as the treatment of choice for lysosomal storage diseases, including Gaucher disease, Fabry disease and attenuated variants of mucopolysaccaridoses type 1 (MPS 1) (17,18). However, the inability of the intravenously administered enzymes to penetrate the blood-brain barrier severely limits the application of this approach for the treatment of other metabolic disorders involving the central nervous system (19,20).

One approach to the delivery of proteins into cells is their fusion with protein transduction domains (PTDs). Most PTDs are cationic peptides (11–34 amino acids) that interact with the negatively charged phospholipids and carbohydrate components of the cell membrane (20–22). When PTDs are fused to other proteins, they enable passage through cell membranes in a fashion yet to be clearly understood but that has been elucidated as non–receptor-mediated, non–clatherin-mediated endocytosis or phagocytosis. The best-known, most used and investigated and well-tested PTD is the transactivator of transcription (TAT) peptide originating from the human immunodeficiency virus (HIV)-1.

TAT is an 11–amino acid (residues 47–57) arginine- and lysine-rich portion of the Tat protein encoded by HIV-1 (19,23). TAT fusion proteins have previously been shown to be rapidly and efficiently introduced into cultured cells, intact tissue and live tissues when injected into mice (20,21,24,25). It was also demonstrated that TAT fusion proteins traverse mitochondrial membranes (26). We proposed a novel but simple approach for the delivery of a wild-type mitochondrial protein/enzyme directly to its subcellular location and into its natural complexes, in the form of a fusion protein for cell-directed protein replacement therapy. Our approach was to fuse the natural/normal protein/enzyme that is mutated in a specific mitochondrial disease with the TAT, which will lead the protein/enzyme into the cells and their mitochondria, where it will substitute for the mutated endogenous protein.

This novel approach was recently tested by using mitochondrial lipoamide dehydrogenase (LAD) deficiency as a model. LAD is the E3 subunit of the three α-ketoacid dehydrogenase complexes in the mitochondrial matrix that are crucial for the metabolism of carbohydrates and amino acids. We constructed and highly purified the TAT-LAD fusion protein and demonstrated that TAT-LAD enters patients' cells rapidly and efficiently, reaching the mitochondria. Inside the mitochondria, TAT-LAD is processed normally and restores LAD activity. Most importantly, we showed that TAT-LAD is able to almost completely restore the activity of the mitochondrial multienzyme pyruvate dehydrogenase complex (PDHC) within the treated patients' cells (15). Moreover, we demonstrated the delivery of TAT-LAD into E3-deficient mice tissues and that a single administration of TAT-LAD results in a significant increase in the enzymatic activity of PDHC within the liver, heart and mainly the brain of TAT-LAD–treated E3-deficient mice (27). These results established for the first time *in vivo,* in an animal model, the proof-of-concept for the feasibility of TAT-mediated protein replacement therapy for mitochondrial diseases.

In this study, we designed and constructed the TAT-C6ORF66 (TAT-ORF) fusion protein. Our results indicate that TAT-ORF is able to enter patients' cells and their mitochondria rapidly and efficiently. TAT-ORF is active and led to an increase in complex I activity. TAT-ORF also increased in the number of patients' cells and improved the activity of their mitochondria. Additionally, we observed an increase in ATP production, a decrease in mitochondrial content and a decrease in the level of reactive oxygen species.

Our results suggest that this approach of protein replacement therapy for the treatment of mitochondrial disorders caused by mutation in the C6ORF66 (NDUFAF4) assembly factor is highly promising.

MATERIALS AND METHODS

Cells

Primary fibroblast cultures from three patients (F528, F511 and F334) of the same family were obtained from forearm skin biopsies (with informed consent). The patients carry a mutation in the second exon of the *C6orf66* (*NDUFAF4*) gene with a T/C substitution at nucleotide 194 that predicts an Leu65Pro mutation. All of the patients were homozygous for the mutation (15). Human primary fibroblasts from healthy donors and from a patient carrying a nonrelevant mutation at the gene coding for the NDUFS2 subunit of complex I were used as controls. Cell cultures were grown in Dulbecco's modified Eagle medium (DMEM), supplemented with

20% fetal calf serum, 1% glutamine and 1% sodium pyruvate at 37°C in an atmosphere of 5% CO_2. All experiments on human primary cultures were performed according to the ethical guidelines of the Hadassah Medical Center.

Construction of Plasmid-Expressing TAT-ORF Fusion Protein

TAT fusion protein was generated by using the pTAT plasmid, provided by Steven F Dowdy (San Diego School of Medicine, San Diego, CA, USA). The plasmid contains six histidines (6xhis tag) followed by the TAT peptide (amino acids 47–57). To construct a plasmid with the C6ORF66 fused to the TAT, the sequence for human precursor C6ORF66 (NDUFAF4) was amplified by polymerase chain reaction (PCR) from a lymphocyte cDNA library from a healthy person, by using the following oligonucleotides: 5′-CGGGATCCGGGAGCA CTAGTGATT-3′ (forward); 5′-CCCCT CGAGTCATTTGATCGTATTGC-3′ (reverse). The PCR product and the pTAT plasmid were cut with *Bam*HI and *Xho*I and ligated, thus producing a plasmid (pHTO) coding for the TAT-ORF fusion protein (HisX6-TAT-C6ORF66; see schematic presentation of the fusion protein in Fig. 1). The clone was confirmed by sequence analysis.

Protein Expression and Purification

Escherichia coli BL21-CodonPlus (λDE3)-competent cells transformed with plasmid encoding the fusion protein were grown at 37°C in a saline lactose broth medium containing kanamycine (50 μg/mL), tetracycline (12.5 μg/mL) and chloramphenicol (34 μg/mL). At an optical density at 600 nm (OD600) of 0.2–0.3, 0.1% glycerol and 0.1 mmol/L potassium glutamate were added to the culture, which was then heat shocked for 20–30 min at 42°C, after which the bacteria were grown at 37°C until an OD600 of 0.8. TAT-ORF fusion protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (0.1 mmol/L, final concentration). After a 24-h incubation at 22°C, the cells were harvested by centrifugation (3,220*g* for 20 min at 4°C). The cell pellet was kept at –80°C for at least overnight. For purification of the TAT-ORF protein, the bacterial cell pellet was dissolved in 20 mmol/L Tris-HCl, pH 7.7, 150 mmol/L NaCl, 0.2 mg/mL lysosyme and 1 mmol/L phenylmethylsulfonylfluoride (Sigma-Aldrich, St. Louis, MO, USA), followed by sonication. The suspensions were clarified by centrifugation (35,000*g* for 30 min at 4°C), and 10 mmol/L imidazole was added to the supernatant containing the fusion protein, which was then loaded onto preequilibrated HiTrap chelating HP columns (Amersham Pharmacia Biotech, Uppsala, Sweden). The columns were washed by stepwise addition of increasing imidazole concentrations. Finally, the target protein was eluted with elution buffer (20 mmol/L Tris-HCl, pH 7.7, 150 mmol/L NaCl, 500 mmol/L imidazole). All purification procedures were carried out by using the FPLC system ÄKTA (Amersham Pharmacia Biotech). Imidazole was removed by dialysis against 20 mmol/L Tris-HCl, pH 7.7, and 150 mmol/L NaCl. Aliquots of the proteins were kept frozen at –80°C until use.

Western Blot Analysis

Proteins (5–20 μg protein/lane) were resolved on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto an Immobilon-P transfer membrane (Millipore, Bradford, PA, USA). Western blot analysis was performed by using anti-C6ORF66 (15), anti-His (Amersham Pharmacia Biotech), anti-α-tubulin (Serotec, Oxford, UK) and anti-E1α (Molecular Probes, Eugene, OR, USA) antibodies.

Isolation of Mitochondria

Mitochondria were obtained through a differential centrifugation: cells were homogenized in buffer A (320 mmol/L sucrose, 5 mmol/L Tris-HCl, 2 mmol/L EGTA, pH 7.4) with a Dounce homogenizer (Teflon glass) and centrifuged for 3 min at 2,000*g* to remove nuclei and cell debris. The supernatant obtained was centrifuged for 10 min at 12,000*g* at 4°C to pellet the mitochondria. The resulting pellet was resuspended in buffer A containing 0.02% digitonin (Sigma-Aldrich) to release trapped mitochondria and then recentrifuged. The mitochondrial pellet was washed again twice with buffer A and kept at –80°C until use. The purity of the subcellular fractions was confirmed by using the specific marker antibodies α-tubulin (cytoplasm) and E1α (mitochondria) (see Western blot analyses for details).

Delivery of the Fusion Protein into Cells and Their Mitochondria

Delivery into patients' cells. Cells were plated on six-well plates or in 250-mL flasks (NUNC Brand Products, Roskilde, Denmark). When the cells reached 90% confluence, the medium was replaced with fresh medium containing 0.02 μg/μL (final concentration) TAT-ORF fusion protein for various time periods. After incubation, the cells were washed with phosphate-buffered saline (PBS), trypsinized, pelleted and kept at –80°C until use. The pellets were resuspended in PBS containing 0.5% Triton X-100 and 1 mmol/L PMSF, kept on ice for 10 min and centrifuged at 15,000*g* for 10 min. The supernatants were analyzed by Western blot.

Delivery into isolated mitochondria. A total of 15 μg purified mitochondria from the cells from patient F528 was incubated with 10.68 μg purified TAT-ORF in buffer A at 30°C for various time periods. After incubation, the mitochondria were centrifuged at 12,000*g* for 10 min at 4°C and washed three times with buffer A. Finally, a mitochondrial lysate was prepared by dissolving the mitochondria in water. The mitochondrial lysate was analyzed by Western blot.

Identification of the TAT-ORF fusion protein within patients cells by confocal microscopy. TAT-ORF fusion protein was labeled with fluorescein isothiocyanate by using a protein-labeling kit (EZ-Label; Pierce Biotechnology, Rockford, IL, USA) in accordance with the manufacturer's instructions. Unbound

fluorescent dye was removed by dialysis against PBS. Cells grown on coverslips to 50–70% confluence were treated with fluorescein isothiocyanate–labeled TAT-ORF (0.1 μg/μL, final concentration) for various time periods at 37°C. After incubation, the cells were washed with PBS and analyzed by using a confocal laser-scanning microscope (Nikon C1; Nikon Instruments, Melville, NY, USA).

Complex I Activity

Complex I activity was determined in isolated mitochondria. A total of 870 μL distilled water was added to 5 μg (protein) of isolated mitochondria (dissolved in 10 μL buffer A, see above), mixed and incubated for 2 min at room temperature to allow osmotic disruption. Subsequently, 125 μL reaction mixture containing the following was added: 80 mmol/L Tris, pH 7.8, 5.2 mg/mL NaN_3, 8 mg/mL fatty acid–free bovine serum albumin, 0.8 mmol/L NADH and 0.4 mmol/L coenzyme Q. Absorbance was measured at 340 nm for 5–10 min, after which 5 μL rotenone (2 mg/mL) was added and the OD at 340 nm was recorded for an additional 5 min. Complex I activity was calculated as ΔOD – ΔODrot × 1,000/6.22 × mg protein and expressed as nmol/min/mg protein. ΔOD indicates the slope of the OD recorded from the point the reaction mixture was added over time. ΔODrot indicates the slope of the OD recorded after adding rotenone.

Cell Viability

F528 cells (5×10^3/100 μL per well) were seeded and treated with TAT-ORF fusion protein (0.02 μg/μL final concentration; however, at different start concentrations), for 24–48 h, after which, CellTiter-Blue reagent (Promega, Madison, WI, USA) was added according to the manufacturer's instructions to determine cell survival. All treatments were performed in triplicate.

Functionality of Mitochondria After Treatment with TAT-ORF

Measurements were performed essentially as described by Golubitzky *et al.* (28). Briefly, 10^4/100 μL F511 cells were seeded in 96-well plates in glucose-rich medium. After 24 h, the medium was replaced with medium lacking glucose, and the cells were incubated for 48 h, after which TAT-ORF (final concentration 0.02 μg/μL) was added for an additional 24 h. Various mitochondrial measurements were performed. The amount of cells was determined by using 1% methylene blue. ATP production was determined by using the ATPlite™ Luminescence Assay System (PerkinElmer, Waltham, MA, USA) according to the manufacturer's recommendations. Free radical levels were measured by dichlorodihydrofluorescein diacetate (DCF) (Biotium, Hayward, CA, USA). The mitochondrial content and mitochondrial membrane potential were estimated as follows: 200 nmol/L MitoTracker green FM (Invitrogen/Life Technologies, Carlsbad, CA, USA; final concentration) was added to the cells and incubated in the dark for 45 min. A total of 50 nmol/L tetramethylrhodamine ethyl ester (TMRE) (final concentration) was then added, and the cells were incubated for an additional 45 min. After incubation, the cells were washed and fluorescence was recorded at 485/590 nm excitation/emission and mitochondrial membrane potential at 485/528 nm excitation/emission. Results were normalized to cell content (methylene blue) or to mitochondrial content (mitotracker green).

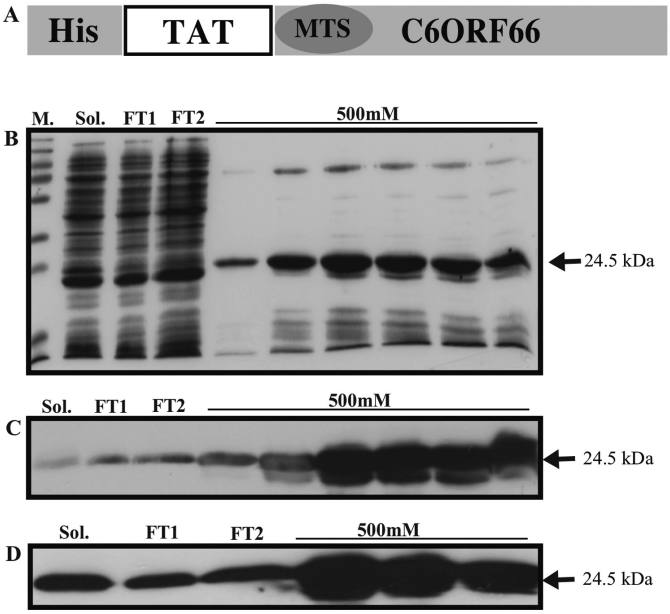

Figure 1. Schematic representation of TAT-ORF and its purification. (A) Schematic representation of TAT-ORF fusion protein. SDS-PAGE (B) and Western blots using anti-His antibodies (C) or anti-C6ORF66 antibodies (D) to analyze the purification steps of TAT-ORF by affinity chromatography as described in Materials and Methods are shown. Arrows indicate the TAT-ORF fusion protein. FT1, flow through 1; FT2, flow through 2; His, 6xhis tag; M., marker; MTS, mitochondrial targeting sequence; Sol., soluble fraction; TAT, Tat peptide.

RESULTS

Construction, Expression and Purification of the TAT-ORF Fusion Protein

To deliver the human C6ORF66 assembly factor into patient cells, we constructed the recombinant fusion protein TAT-ORF. We used the sequence of the human full-length ORF containing the first 34 amino acids predicted as the MTS (15). The fusion protein was constructed with a histidine tag (6xhis tag) at its N′-terminus. Figure 1A represents the schematic structure of TAT-ORF. The protein was expressed and highly purified using affinity chromatography. The solu-

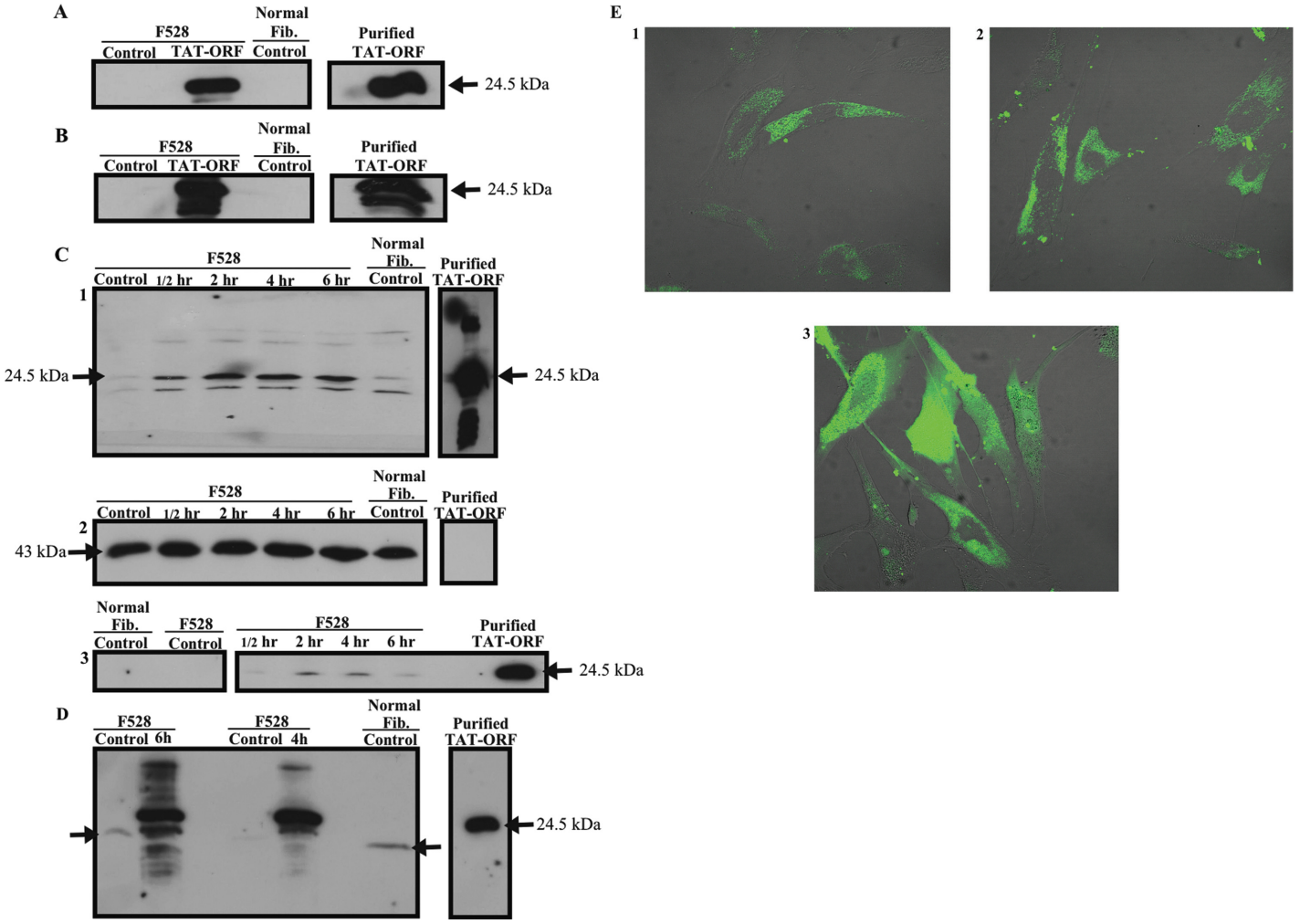

Figure 2. Internalization of TAT-ORF into isolated mitochondria and cells of F528 patients. (A, B) Internalization of TAT-ORF into mitochondria isolated from cells of F528 patients. Isolated mitochondria were added with TAT-ORF (0.11 μg/μL; final concentration) for 2 h, washed and submitted to Western blot analysis by using anti-His (A) (1:30,000 dilution) or anti-ORF (B) (1:10,000 dilution) antibodies. (C, D) Internalization of TAT-ORF into cells from F528 patients. F528 cells were treated with TAT-ORF (0.02 μg/μL; final concentration) for various time periods. The cells were washed and mitochondria were isolated and submitted to Western blot analysis using anti-ORF (C1 1:50,000 dilution), anti-E1α (C2; 1:1,000 dilution) or anti-His (C3; 1:10,000 dilution) antibodies. (D) Repeating the experiment with a higher concentration of the fusion protein added to the F528 cells (0.1 μg/μL, final concentration) and loading twice the amount of purified mitochondria (25 μg protein per lane), from both F528 treated cells and normal fibroblast. Proteins were identified by using anti-ORF antibodies. Arrows indicate the TAT-ORF fusion protein (A–C1, 3; D, right image), the E1α (C2) or the endogenous C6ORF66 protein (D, left image). (E) After internalization of FITC-labeled TAT-ORF into F528 cells for 1 h (E1), 2 h (E2) and 3 h (E3), by using confocal microscopy. Magnification 60×.

ble fraction was loaded on a Ni-chelating column, followed by multiple washing steps with increasing concentrations of imidazole and finally eluted at a high concentration of imidazole (Figure 1B). SDS-PAGE analysis and Western blotting, by using anti-ORF (Figure 1C) or anti-His (Figure 1D) antibodies, confirmed the identity of the highly purified fusion protein.

TAT-ORF Is Delivered into Isolated Mitochondria and Patients' Cultured Cells

To examine the ability of TAT-ORF to be delivered across the inner mitochondrial membrane, we first incubated TAT-ORF with isolated mitochondria from cells from patient F528 for 2 h. Mitochondria isolated from normal, control fibroblasts served as a control. After incubation, the mitochondria were treated with proteinase K to digest proteins nonspecifically adsorbed to the outer membrane, thereby ensuring that the mitochondrial extract contained only proteins within the mitochondria. As seen in Figures 2A and B, after treatment, the TAT-ORF was detected within the mitochondria. However, it should be noted that the endogenous mutated ORF protein was not detected in

this Western blot, neither in mitochondria isolated from F528 cells nor from normal, control fibroblasts, probably because of a low expression of the endogenous protein (see below).

Next we examined the ability of TAT to deliver the human ORF assembly factor into patients' cells and their mitochondria by incubating F528 cells for various time periods with the purified TAT-ORF fusion protein. After incubation, mitochondria were prepared and analyzed by Western blot, by using anti-ORF, anti-His and anti-E1α antibodies. As shown in Figure 2C, the TAT-ORF fusion protein (24.5 kDa) rapidly entered the cells and was already detectable after 30 min of incubation. A steady state was reached after 2 h, which remained stable for at least 6 h (Figure 2C1). As was the case with the isolated mitochondria, the endogenous ORF protein was not detected in the patients' cells or in the control fibroblasts. However, by repeating the experiment with a higher concentration of the fusion protein added to the cells (0.1 μg/μL, final concentration) and loading twice the amount of the purified mitochondria from both F528-treated cells and normal fibroblasts, a faint band corresponding to the endogenous ORF protein was observed in the control fibroblasts (Figure 2D). Incubation of the patients' cells with the fusion protein for 6 h revealed that it is partially processed into its natural size (see Figure 2D, arrows). We also followed the fate of TAT-ORF upon incubation of F528 cells with fluorescein isothiocyanate (FITC)-labeled TAT-ORF for various time periods, using confocal microscopy. As seen in Figure 2E1–3, green FITC staining within the cells can be detected as soon as 1 h after incubation, and its intensity increased with time. Thus, TAT-ORF fusion protein enters patients' cells and their mitochondria rapidly and efficiently.

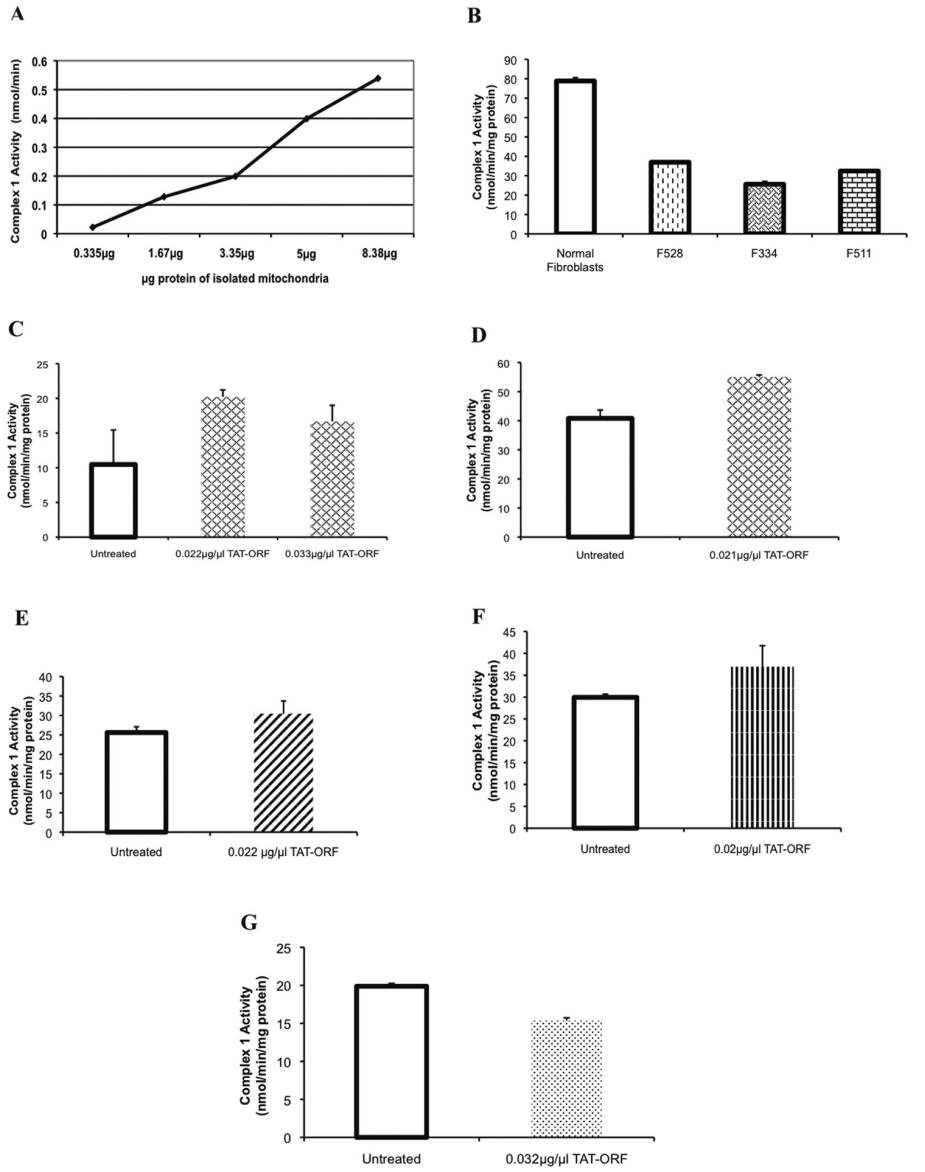

Figure 3. Complex I activity in various cells. (A) Complex I activity in healthy fibroblasts as a function of amount (in micrograms protein added) of mitochondria added. (B) Complex I activity in mitochondria isolated from patient cells mutated in C6ORF66; data for F528, F334 and F511 cells, as compared with activity in mitochondria isolated from healthy fibroblasts, are shown. The effect on complex I activity is shown for TAT-ORF added to isolated mitochondria from F528 cells (C). TAT-ORF was added to patients' cells: F528 (D), F334 (E), F511 (F) and nonrelevant NDUFS2 cells (G); mitochondria was isolated and complex I activity was measured. TAT-ORF was added at a final concentration of 0.022 μg/μL (C–F) or 0.032 μg/μL (G), for 3 h (C) or 24 h (D–G). Complex I activity was measured as described in Material and Methods. Activity values are expressed as nmol/min/mg protein and presented as average of triplicates ± standard deviation (SD).

TAT-ORF Restores Complex I Activity in Patients' Cells and Its Activity Is Specific

We first calibrated the enzymatic assay for measuring complex I activity in isolated mitochondria from control fibroblasts. As seen in Figure 3A, activity of complex I increases with the amounts (in micrograms protein) of mitochondria added, thus validating the enzymatic assay.

Because C6ORF66 (NDUFAF4) is an assembly factor of complex I (15), we tested the activity of complex I in mitochondria isolated from cultures of pa-

tients' cells, compared with its activity in mitochondria isolated from control fibroblasts. We tested three cell cultures established from three individuals of the same family, carrying the same mutations (cell cultures F528, F334 and F511). Indeed, all three patients' cell cultures had impaired complex I activity, although some variability in the enzymatic activity values was observed (Figure 3B).

To test the biological activity of TAT-ORF in patients' cells, isolated mitochondria from F528 cells were directly incubated with TAT-ORF fusion protein for 3 h, and complex I activity was measured. As demonstrated in Figure 3C, TAT-ORF fusion protein increased the activity of complex I by 59–92%, depending on the amounts of the fusion protein added.

Next TAT-ORF fusion protein was added to cultured F528 cells for 24 h; mitochondria were then isolated and complex I activity was measured. TAT-ORF fusion protein restored the activity of complex I by 46% in patient cells (Figure 3D). The activity of TAT-ORF fusion protein was tested on the additional patient cells. Incubation with TAT-ORF (24 h, 0.022 μg/μL final concentration) increased complex I activity by ~20% in F334 cell cultures (Figure 3E) and by 23% in F511 cells (Figure 3F). Thus, TAT-ORF fusion protein improves complex I activity in patient cells with mutations in the C6ORF66 assembly factor.

To assure that activity of TAT-ORF fusion protein specifically improves cells mutated in C6ORF66 (NDUFAF4), NDUFS2 patient cells were incubated with the fusion protein, and complex I activity was measured. NDUFS2 patient cells carry a mutation in the NDUFS2 protein, one of the subunits of complex I. Because these cells carry a different mutation in a gene encoding a structural complex I subunit, incubation with TAT-ORF, as expected, did not increase complex I activity (Figure 3G). Thus, TAT-ORF fusion protein specifically acts on cells mutated in the NDUFAF4 assembly factor.

To test whether TAT-ORF has any toxic effect, F528 patient cells were incubated

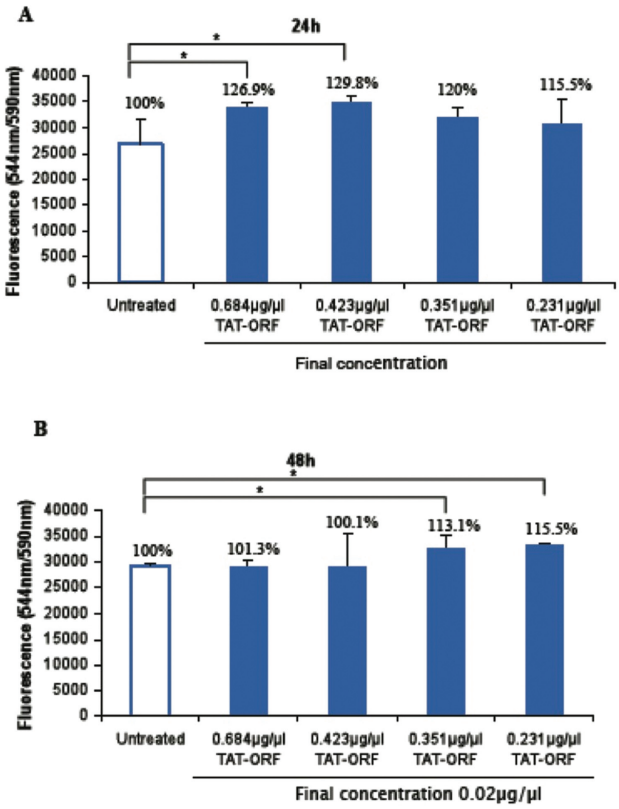

Figure 4. Effect of TAT-ORF fusion protein on patients' cell viability. F528 cells were incubated with TAT-ORF (0.02 μg/μL final concentration; however, at different start concentrations) for 24 h (A) or 48 h (B), and cell viability was measured. Results are expressed as percentage of proliferation (untreated cells being 100%) and are the mean of triplicates ± SD. *$p < 0.05$.

with various start concentrations (see below) of the fusion protein, for 24 or 48 h, and cell viability was determined. As seen in Figure 4, TAT-ORF is not toxic to patient cells, but rather improved its viability.

Nevertheless, TAT-ORF fusion protein might be toxic to normal cells or at least interfere with normal complex I activity; thus, TAT-ORF was added to normal fibroblasts isolated from a healthy donor followed by mitochondria isolation and complex I measurements. Incubation with TAT-ORF had no effect on complex I activity (results not shown) in the normal cells. Moreover, TAT-ORF also had no effect on the activity of complex II in these normal cells (results not shown). Thus, TAT-ORF is nontoxic to normal cells.

Because TAT-ORF lacks any known enzymatic activity by itself and is not present in the final respiratory chain complex, many aspects regarding the fusion protein preparation and the assay variables (amounts of mitochondria, time dependency, start protein concentration and its final protein concentration in the assay) needed to be calibrated to achieve the optimal conditions to restore complex I activity. All these experiments were performed with the cells from patient F528. We found that 1.67–3.35 μg protein of mitochondria should be used in the assay (Figure 5A1–3). The TAT-ORF fusion protein should be incubated with the mutant cells for at least 24 h (Figure 5B1–3). The start concentration of the highly purified TAT-ORF fusion protein should not be higher than 0.5 μg/μL (Figure 5C), and its final concentration in the assay should be ~0.02 μg/μL (Figure 5D). Under these conditions, TAT-ORF fusion protein increased complex I activity by 20–50%.

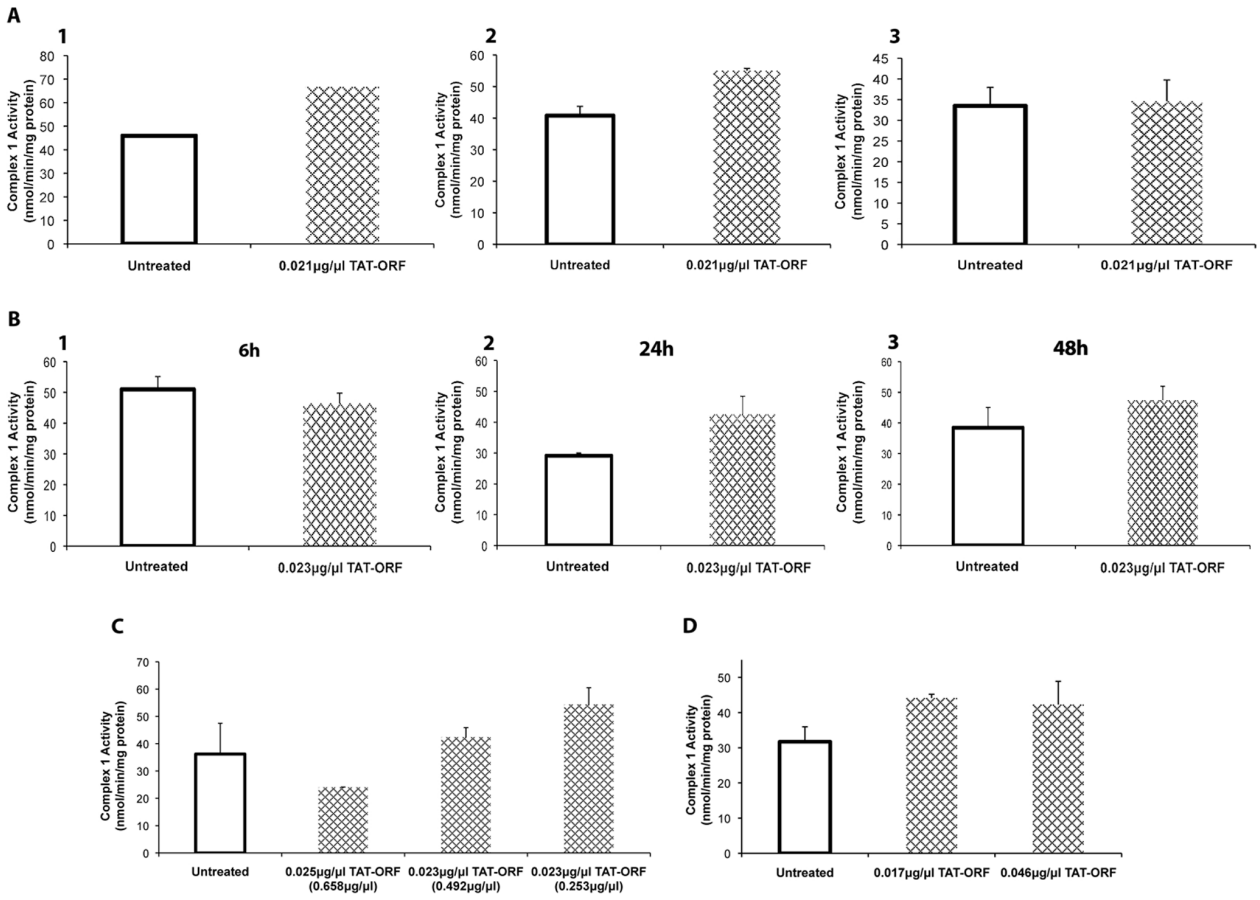

Figure 5. Complex I activity in TAT-ORF–treated cells: calibration studies. (A, B) Complex I activity in F528 cells treated with TAT-ORF fusion protein (0.022 µg/µL, final concentration) as a function of the amount of mitochondria (A): 1.67 µg protein (1), 3.35 µg (2), 5 µg (3) of isolated mitochondria and incubation time (B). (C,D) Complex I activity in F528 cells treated with TAT-ORF fusion protein as a function of the start concentration of the TAT-ORF fusion protein (C) and final concentration in the assay (D). Incubation was performed for 24 h. Complex I activity was measured as described in Material and Methods. Activity values are expressed as nmol/min/mg protein and presented as average of triplicates ± SD.

TAT-ORF Improves Functionality of Mitochondria from Patient Cells

One important question regarding the biological activity of TAT-ORF fusion protein is whether it will improve not only the activity of complex I per se but also the functionality of the whole mitochondria of the treated cells. For this purpose, F511 cells were treated with TAT-ORF under the optimized conditions described above, and its effect was tested on the various mitochondrial parameters. As demonstrated in Figure 6, TAT-ORF treatment increased proliferation of cells in glucose-free medium by 25% compared with nontreated patient cells (Figure 6A), whereas mitochondrial content decreased by 23% in the treated cells (Figure 6B). A similar increase in proliferation was observed when F528 cells were treated with TAT-ORF under similar conditions (Figure 4). Total production of ATP in the treated cells did not change (Figure 6C); however, ATP levels normalized to mitochondrial content increased by 25% (Figure 6D). No effect on mitochondrial membrane potential was observed (Figure 6E); however, free radical levels decreased by ~25% in the treated cells (Figure 6F). Thus, treatment with TAT-ORF significantly improved the mitochondrial functionality of patient-derived cells, harboring a mutation in the C6ORF66 (NDUFAF4) assembly factor.

DISCUSSION

Mitochondrial complex I is vital to the supply of energy in the body. Disturbances in complex I function can result in complex I deficiency, which is the most common biochemical defect of the OXPHOS system. Moreover, although complex I dysfunction initially attracted attention in the context of inherited metabolic disorders, it is now increasingly implicated in diabetes and neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease (29–31).

Currently, there is no effective cure for genetic mitochondrial metabolic disorders. Although a few strategies for mitochondrial dysfunction have been de-

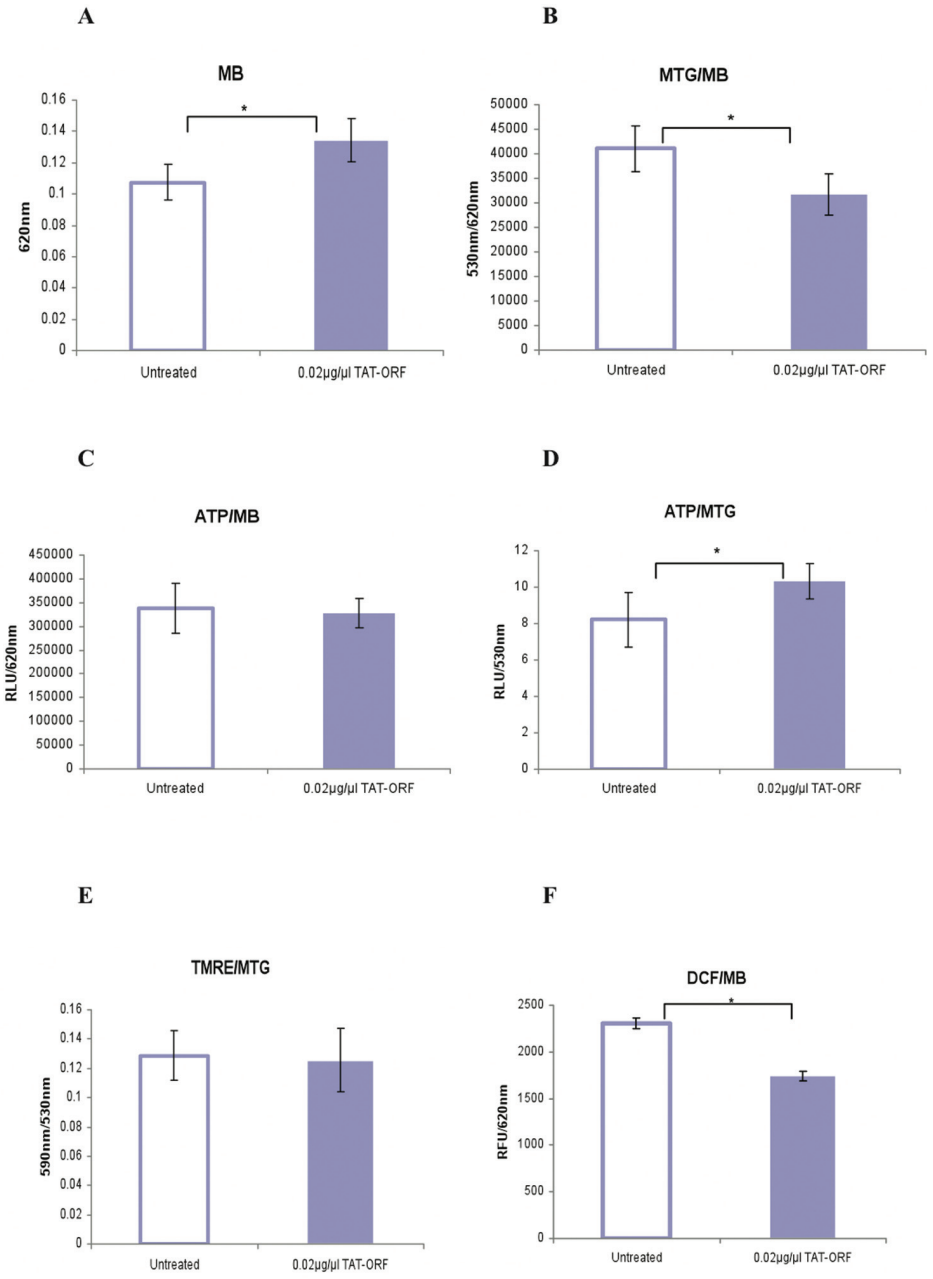

Figure 6. Treatment with TAT-ORF improves functionality of patients' mitochondria mutated in the C6ORF66 (NDUFAF4) assembly factor. The effect of TAT-ORF treatment was tested on the following: amount of the cells (A); mitochondrial content (B); production of total ATP in the cells, normalized to the amount of cell ATP/methylene blue (MB) (C) and normalized to mitochondrial content ATP/MitoTracker Green (MTG) (D); mitochondrial membrane potential TMRE/MTG (E); and production of free radicals DCF/MTG (F). The experiment was performed with cells from patient F511 treated with TAT-ORF (0.022 µg/µL, final concentration) for 24 h. All measures were performed as described in Materials and Methods. Data are presented as the average of four to five repeats ± SD. *$p < 0.5$.

scribed, most cases are not fully treatable. We chose to focus on complex I deficiency caused by mutation in the assembly factor C6ORF66 (NDUFAF4). Our goal was to further establish the approach to treat mitochondrial disorders using cell- and organelle-directed protein/enzyme replacement therapy with TAT peptide as the moiety delivery system.

We designed and constructed the TAT-ORF fusion protein by gene fusion techniques. We expressed TAT-ORF in an *E. coli* expression system and highly purified it. Our results indicate that TAT-ORF enters patient cells and their mitochondria rapidly and efficiently. TAT-ORF is biologically active and led to an increase in complex I activity. TAT-ORF also increased the number of patient cells and improved the activity of their mitochondria. Additionally, we observed an increase in ATP production, a decrease in mitochondria content and a decrease in the level of reactive oxygen species.

Normal activity of complex I in healthy fibroblasts ranges between 45 and 80 mmol/min/mg protein. In contrast, activity of complex I in the patient cells carrying a mutation in C6ORF66 (F528, F334, F511) is 25–50 mmol/min/mg protein (Figure 3B). Treatment with TAT-ORF increased complex I activity in patient cells by 20–50% compared with nontreated cells (Figures 3 and 5). However, it should be pointed out that complex I has a molecular mass of 1 MDa, being the largest enzyme of the mitochondrial OXPHOS system. Thus, it may be that the amount of the numerous other complex I subunits is not sufficient to attain maximum activity. Being such a large complex, intensive efforts are being undertaken to study the assembly pathway of the holoenzyme complex; however, little is known regarding the complex half-life or the expression of the individual complex I subunits. The effect of long-term treatment with TAT-ORF fusion protein on the number of integral subunits in complex I should be investigated.

However, another important point is that, in these types of metabolic disorders and especially mitochondrial disorders, there is no need to restore enzyme activity back to 100%. It is enough to raise the level above a certain energetic threshold, which can vary from patient to patient depending on the basal enzymatic activ-

ity. Hence, the delivery of sufficient amounts of the wild-type protein (in this case, the ORF assembly factor) may be surprisingly easy to achieve, thus improving the overall condition of the patients. Indeed, treatment with TAT-ORF fusion protein not only increased complex I enzymatic activity, but most importantly, improved functionality of the mitochondria of patients' treated cells, as well as the growth of the cells (Figures 4 and 6). Thus, once again, as in the case of TAT-LAD (15,27), TAT-mediated replacement of one mutated component (in this case, a factor necessary for the assembly of the huge complex) restores the activity of an essential mitochondrial multicomponent enzymatic complex in cells of patients with a protein deficiency.

Using TAT-mediated protein delivery in protein replacement therapy has many advantages; however, this system still has some drawbacks. The most important drawback to be considered is the possible immunogenicity of TAT fusion proteins. In an earlier report, TAT failed to enhance the immunogenicity of cytomegalovirus (CMV) nucleoprotein in mice when only a single booster dose was given (32). Nevertheless, it was speculated that TAT fusion proteins, especially in the context of repeated dosing, as required in many protein replacement therapies, could elicit an immunogenic response such as the production of neutralizing antibodies. We believe that the rapid intracellular transduction of TAT fusion proteins would protect it from exposure to the immune system. This issue was addressed in an important *in vivo* study, where a human purine nucleoside phosphorylase (PNP) was given to PNP knockout mice ($PNP^{-/-}$) in the form of TAT-PNP (33). The immuno-response was measured by the presence of specific IgG antibodies after 2–3 wks of treatment with TAT-PNP compared with treatment with the nonfused PNP. The authors detected the same maximal levels of specific antibodies against the human PNP, either given as TAT-PNP or as nonfused PNP. These findings suggest that the immune response observed in TAT-PNP–treated mice was not against TAT itself, but rather against the xenogeneic human PNP protein (33). Thus, TAT itself did not increase the immunogenicity of the human protein.

In addition, the rapid intracellular transduction protected TAT-PNP from neutralizing antibodies, and despite the development of anti-PNP antibodies during treatment, TAT-PNP maintained its biological activity within all organs. Moreover, in a recent report, various PTDs were tested, including the TAT peptide, and were found to be nontoxic and nonimmunogenic *in vitro* at 10 and 5 μmol/L concentrations and *in vivo* at a dose of 5 mg/kg. This result suggested that these PTDs have great potential as a system to deliver therapeutic molecules into the cell without risks of toxicity and inflammatory reactions (34).

PTDs, such as TAT, have the ability to penetrate the plasma membrane as well as the mitochondrial membranes (26,35,36), leading to the possible application of the PTD technology to a protein-therapeutic approach for mitochondrial disorders. Besides our research regarding the TAT-LAD fusion protein (see the Introduction), a number of studies have been reported.

Wilson's group (37) used an MTS (from the human manganese superoxide dismutase [MnSOD] mitochondrial protein) to target the DNA repair enzyme exonuclease III (ExoIII) into the mitochondria of breast cancer cells by using the PTD technology. The produced recombinant MTS-ExoIII-TAT fusion protein was successfully targeted into the mitochondria of cells, where it played a crucial role in the survival of these cells. TAT protein replacement therapy was also reported by Foltopoulou *et al.* (38) for a mitochondrial respiratory chain disorder, fatal infantile cardioencephalomyopathy, and for COX deficiency due to mutations of the *Sco2* gene. Human *SCO2* is a nuclear gene encoding the full-length L-Sco2 protein (266 amino acids), a COX assembly protein. Human recombinant TAT-L-Sco2 fusion protein was successfully internalized into mammalian cells and translocated into its mitochondria (39). By using imatinib-treated human CML K-562 erythroleukemia cells, with low levels of SCO2 mRNA and COX deficiency primary fibroblasts from SCO2/COX-deficient patients, it was demonstrated that, in both cell cultures, adding recombinant fusion TAT-L-Sco2 protein resulted in enzymatic restoration of COX activity (38).

Recently, Payne's group reported the use of the TAT-frataxin (TAT-FRN) fusion protein for the treatment of Friedreich ataxia (FRDA). They demonstrated that TAT-FRN binds iron *in vitro*, transduces into mitochondria of FRDA-deficient fibroblasts and reduces caspase-3 activation in response to exogenous iron-oxidant stress. Injection of TAT-FRN protein into mice with a conditional loss of FRN increased their growth velocity and mean lifespan by 53%, increased their mean heart rate and cardiac output, increased activity of aconitase and reversed abnormal mitochondrial proliferation and ultrastructure in the heart (39).

Our results suggest that this approach of protein replacement therapy for the treatment of mitochondrial disorders caused by mutation in the C6ORF66 (NDUFAF4) assembly factor is very promising. We believe that together with results obtained in our laboratory with regard to LAD deficiency and results of others, this approach will revolutionize the management of these types of mitochondrial and metabolic disorders in modern medicine.

CONCLUSION

Replacement of the C6ORF66 assembly factor (NDUFAF4) by the TAT-ORF fusion protein restores complex I activity in cells derived from patients. TAT-ORF fusion protein is delivered into the cells and their mitochondria rapidly and efficiently, where it is most probably processed, restoring complex I activity and, most importantly, improving the functionality of the treated mitochondria. Additionally, treatment increases ATP production in the mitochondria and de-

creases the number of mitochondria as well as the level of reactive oxygen species. The success of this TAT-mediated replacement protein therapy approach and the fact that the replacement of an assembly factor of a multicomponent complex can actually restore the activity of a whole machine, even a complex one, makes this approach promising.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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