The role of the LRPPRC (leucine-rich pentatricopeptide repeat cassette) gene in cytochrome oxidase assembly: mutation causes lowered levels of COX (cytochrome c oxidase) I and COX III mRNA

Fenghao XU*, Charles MORIN†, Grant MITCHELL‡, Cameron ACKERLEY§ and Brian H. ROBINSON* || 1

*Metabolism Research Programme, The Research Institute, The Hospital for Sick Children, 555 University Ave., Toronto, ON, Canada M5G 1X8, †Department of Pediatrics and Clinical Research Unit, Hôpital de Chicoutimi, 305 St-Vallier, Chicoutimi, QC, Canada G7H 5H6, §Service de Génétique Medicale, Hôpital Sainte-Justine, 3175 Côte Sainte-Catherine, Montréal, QC, Canada H3T 1C5, |Department of Pediatric Laboratory Medicine, The Hospital for Sick Children, 555 University Ave., Toronto, ON, Canada M5G 1X8, and ||Department of Biochemistry, University of Toronto, Medical Sciences Building, 1 King’s College Circle, Toronto, ON, Canada M5S 1A9

Leigh syndrome French Canadian (LSFC) is a variant of cytochrome oxidase deficiency found in Québec and caused by mutations in the LRPPRC (leucine-rich pentatricopeptide repeat cassette) gene. Northern blots showed that the LRPPRC mRNA levels seen in skeletal muscle > heart > placenta > kidney > liver > lung = brain were proportionally almost opposite in strength to the severity of the enzymic cytochrome oxidase defect. The levels of COX (cytochrome c oxidase) I and COX III mRNA visible on Northern blots were reduced in LSFC patients due to the common (A354V, Ala54 → Val) founder mutation. The amount of LRPPRC protein found in both fibroblast and liver mitochondria from LSFC patients was consistently reduced to < 30% of control levels. Import of [35S]methionine LRPPRC into rat liver mitochondria was slower for the mutant (A354V) protein. A titre of LRPPRC protein was also found in nuclear fractions that could not be easily accounted for by mitochondrial contamination. [35S]Methionine labelling of mitochondrial translation products showed that the translation of COX I, and perhaps COX III, was significantly reduced in the presence of the mutation. These results suggest that the gene product of LRPPRC, like PET 309p, has a role in the translation or stability of the mRNA for mitochondrially encoded COX subunits. A more diffuse distribution of LRPPRC in LSFC cells compared with controls was evident when viewed by immunofluorescence microscopy, with less LRPPRC present in peripheral mitochondria.

Key words: cytochrome c oxidase I (COX I) and COX III mRNA, cytochrome oxidase, Leigh syndrome, leucine-rich pentatrico- peptide repeat cassette (LRPPRC) protein, mitochondria.

INTRODUCTION

The LRPPRC (leucine-rich pentatricopeptide repeat cassette) motif containing LRPPRC protein encoded on chromosome 2 is a distant homolog of the yeast protein PET 309p, a protein required for efficient expression of the COX (cytochrome c oxidase) complex [1–3]. The LRPPRC gene was identified as causative for the LSFC (Leigh syndrome French Canadian variant, MIM220111) due to a founder mutation (C1119 → T transition) in exon 9 predicting a missense A354V (Ala354 → Val) founder mutation. The amount of LRPPRC protein found in both fibroblast and liver mitochondria from LSFC patients was consistently reduced to < 30% of control levels. Import of [35S]methionine LRPPRC into rat liver mitochondria was slower for the mutant (A354V) protein. A titre of LRPPRC protein was also found in nuclear fractions that could not be easily accounted for by mitochondrial contamination. [35S]Methionine labelling of mitochondrial translation products showed that the translation of COX I, and perhaps COX III, was significantly reduced in the presence of the mutation. These results suggest that the gene product of LRPPRC, like PET 309p, has a role in the translation or stability of the mRNA for mitochondrially encoded COX subunits. A more diffuse distribution of LRPPRC in LSFC cells compared with controls was evident when viewed by immunofluorescence microscopy, with less LRPPRC present in peripheral mitochondria.

Abbreviations used: COX, cytochrome c oxidase; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; LRPPRC, leucine-rich pentatricopeptide repeat cassette; LSFC, Leigh syndrome French Canadian; NP40, Nonidet P40.

1 To whom correspondence should be addressed, at Metabolism Research Programme, The Research Institute, The Hospital for Sick Children, 555 University Ave., Toronto, ON, Canada M5G 1X8 (email bhr@sickkids.ca).
is associated with lower detectable levels of the COX mRNA transcripts derived from mitochondrial DNA.

**MATERIALS AND METHODS**

**Tissue culture**
All fibroblast cell lines were grown in Dulbecco’s modified Eagle’s medium with glucose (4.5 g/l), glutamine (2.5 mM), penicillin (100 µg/ml) and streptomycin (100 µg/ml) at 37 °C with 5 % CO₂.

**Isolation of mitochondria**
Fibroblast cells were harvested by trypsin and washed twice with ice-cold PBS, and the mitochondria were prepared as described previously [14]. Human liver mitochondria were prepared from autopsy samples obtained with informed consent. Briefly, a 0.5 g of liver sample was minced and homogenized with a polytron homogenizer in mitochondrial preparation buffer [0.34 M sucrose, 100 mM KC1, 10 mM Tris (pH 7.4) and 1 mM EDTA]. After the removal of debris by centrifugation at 1023 g for 10 min, the mitochondrial fraction was prepared as described above [15].

**Nuclear protein preparation**
Fibroblast cells (10 millions) were suspended in a low-salt NP40 (Nonidet P40) buffer [10 mM Hepes (pH 7.9), 10 mM KC1, 1.5 mM MgCl₂, 0.1 % NP40 and 0.5 mM DTT (dithiothreitol) plus a cocktail of proteinase inhibitors] and incubated on ice for 5 min to break the cell membrane. After 1 min of vortex-mixing to strip off the cytoplasmic and organellar fractions, the nuclei in the insoluble fraction were pelleted by centrifuging and washed twice with the same buffer and then suspended in 50 µl of high-salt nuclear extraction buffer [20 mM Hepes (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 25 % glycerol and 0.5 mM DTT plus a cocktail of proteinase inhibitors]. After rocking vigorously at 40 °C for 15 min on a shaking platform, the nuclear protein in the insoluble fraction was collected by centrifugation at 23 426 g for 10 min.

**Northern-blot analysis**
A filter containing RNA samples from different tissues was purchased directly from Clontech (Palo Alto, CA, U.S.A.), whereas the filter containing RNA samples from both the normal and the patient was prepared as follows. Briefly, total RNA was isolated from fibroblast cell lines by using an RNA Easy kit from Qiagen. All hybridization and washing procedures were performed at 68 °C. The washed filter was used to expose an X-ray film at 22 °C for 2 days [17].

**35S-labelling of mitochondrial translation products**
Labelling was carried out by the method of Chomyn [17]. Cultured fibroblasts were passaged for 48 h before pulse labelling in the presence of emetine. Mitochondria were then isolated as described above, and mitochondrial protein were separated by electrophoresis on SDS/polyacrylamide gels (10–20% linear gradient). After electrophoresis, the separated samples were transferred on to a nitrocellulose membrane and exposed to an X-ray film at room temperature (22 °C) for 2 days [17].

**Import of 35S-labelled LRPPRC into rat liver mitochondria**
Full-length LRPPRC cDNA was amplified by reverse transcriptase–PCR, subcloned into TA cloning vector (Invitrogen) and sequenced. The vectors were linearized, purified and quantified. The linearized vector (10 µg) was used to generate [35S]methionine-labelled protein via transcription and translation in vitro by using TNT T7 coupled reticulocyte kit (Promega) according to the manufacturer’s instructions. Label efficiency was determined on an SDS/10% polyacrylamide gel. Import reaction was initiated by adding 50 µl of the translated products in equal volume of freshly isolated rat liver mitochondria (100 µg) in 2x import buffer [20 mM Hepes/KOH (pH 7.5), 1 mM EGTA, 140 mM sucrose, 440 mM mannitol, 66 mM glutamate and 4 mg/ml fatty acid-free BSA] at 30 °C. The reaction was terminated at various times as required (10–60 min) by adding a 10-fold volume of ice-cold import buffer and quickly centrifuging at 23 000 g for 10 min at 4 °C to remove all un-imported protein. After another washing, the pellet was heated in 50 µl of SDS loading buffer at 95 °C for 8 min and 25 µl of each sample was loaded and separated on an SDS/10% polyacrylamide gel. After fixing and drying, the gel was exposed to an X-ray film.

**Immunofluorescence staining of cells**
Mito-tracker red fluorescent protein (Mito-RFP) vector was purchased from Clontech and used to transfect cells from both the normal individual and the patient using SuperFect Transfection reagent from Qiagen. After 2 weeks of selection in G418 medium, the cells were seeded in lab-ték chamber slides (Nalge Nunc International, Rochester, NY, U.S.A.) and cultured overnight. The cells were then fixed, permeabilized and immunostained by using Cytofix/Cytoperm kit from PharMingen (Mississauga, ON, Canada). Briefly, the cells were washed twice with ice-cold PBS and fixed in Cytofix/Cytoperm solution at 4 °C for 20 min. The fixed cells were washed twice with Perm/Wash solution and then incubated in 1:1000 diluted goat anti-rabbit IgG conjugated with horseradish peroxidase, followed by development with ECL® solution (Amersham Biosciences) [16].

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with Perm/Wash buffer and then incubated with FITC-conjugated goat ant-rabbit antibody for another hour. Unbound secondary antibody was removed by repeated washing. After draining away any water remaining on the slide at room temperature in the dark, the wet slide was mounted in SlowFade Antifade with DAPI (4,6-diamidino-2-phenylindole) solution (Molecular Probes). DAPI-stained nuclei, mito-RFP-labelled mitochondria and immunostained LRPPRC were imaged using a confocal fluorescence microscope with appropriate light filters.

**RESULTS**

The presence of a homozygous mutation (C<sup>1119</sup>→T transition) in the LRPPRC cDNA in the majority of the LSFC patients produces a situation where the level of measurable cytochrome oxidase activity in fibroblasts is 50%, the level in liver and brain is 20% or less, but the level in kidney, skeletal muscles and heart is close to normal [12,13]. We performed Northern blotting for LRPPRC mRNA using a variety of human tissue RNA preparations (Figure 1). It is clear that the LRPPRC mRNA level is highest in skeletal muscles > heart > placenta > kidney > liver > lung = brain, reciprocal/opposite almost to the location of the enzymic COX defect in LSFC-COX deficiency and the organs showing significant pathology, i.e. brain and liver. It is also perhaps of interest that patients with LSFC-COX deficiency who die early often enter a phase of decompensation with metabolic acidosis and pulmonary oedema, a situation which may implicate lung as an organ with a metabolic problem, i.e. correlating with low expression of LRPPRC mRNA [12]. There appears to be three transcripts in all tissues, one of 7 kb and the others 5.0 and 4.7 kb respectively (Figure 1). The significance of these three transcripts at present is not clear. Separate Northern blots with probes to the 5'- and 3'-end respectively of the LRPPRC cDNA also displayed three bands (results not shown).

The mRNA expression pattern of mitochondrially encoded COX subunits I, II and III investigated by Northern blotting showed a pattern with the expression order being heart > liver > skeletal muscle > kidney > brain = placenta > lung (Figure 1). The effects of the LSFC mutation on the levels of LRPPRC and COX subunit mRNAs were then examined in fibroblasts from patients by Northern blotting to compare with control samples (Figure 2). Expressions of COX I and COX III were decreased in comparison with the level of β-actin mRNA, a nuclear-encoded cytoskeletal component, and ND1, an mtDNA-encoded transcript for complex I. Ratios of the expressed transcript by densitometry were 21.8, 47.0 and 30.1% for COX I, COX II and COX III transcripts with respect to control when actin transcript density was used as a baseline. Cardiac and skeletal muscles show extra bands at 1.6 kb for actin due to cross-reactivity of the β-actin (ACTB) probe with the myofibrillar form of actin, which is ignored in the calculations [18]. Comparison of transcript density showed that COX I and COX III mRNAs, in particular, were decreased in cells from patients. Immunoblotting showed that LRPPRC protein was greatly decreased in mitochondria by the presence of the mutation to < 25% of the wild-type level in both fibroblasts and liver (Figures 3A and 3B). Compared with the 49 kDa subunit of complex I, the level was undetectable in two out of three samples of liver obtained at autopsy. To investigate import and processing, [14<sup>S</sup>]methionine-labelled LRPPRC protein was prepared by translation in a reticulocyte lysate and incubated with freshly isolated rat liver mitochondria. After gel separation, the labelled pre-LRPPRC precursor protein decreased and an LRPPRC band of lesser molecular mass became dominant over time (Figure 3C). When an A354V mutant transcript was translated, the processing to this lower molecular mass product was slower, indicating decreased import and cleavage of the mitochondrial targeting sequence.

A nuclear fraction of the fibroblasts was prepared by detergent extraction of cells and differential centrifugation, and the level of LRPPRC protein was visualized by immunoblotting (Figure 4). Histone H1 immunoblots show that the level of this protein is the same in all nuclear fractions, whereas there appeared to be LRPPRC protein in the nucleus or at least in a fraction of the cells that is resistant to NP40 extraction. Surprisingly, there was detectable LRPPRC in the nuclear fractions of controls but less in LSFC fibroblasts. Antibody titres for the 49 kDa subunit of...
complex I were very low, but histone H1 was equivalent in controls and patients.

To ascertain whether the lower amount of LRPPRC protein influenced translation of COX mRNAs in mitochondria, the appearance of \(^{35}\)S\(\)methionine-labelled protein in mitochondria was assessed by incubating cultured fibroblasts with \(^{35}\)S\(\)methionine in the presence of emetine. When this was done, a very specific reduction in the amount of labelled COX I and COX III proteins was seen compared with all other proteins (Figure 5). In addition, a prominent extra protein band was seen below the complex I subunit, MTND6. Since this was not visible in control skin fibroblasts, we presumed that this was probably a degradation product of labelled but unassembled COX protein.

Differential intracellular localization of LRPPRC protein was investigated in LSFC fibroblasts by comparing the image obtained with mitochondrially targeted red fluorescent protein with the immunofluorescence (green) image obtained with the LRPPRC

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**Figure 3** Western-blot analysis of LRPPRC contents of skin fibroblasts and liver mitochondria from LSFC patients and controls

The mitochondrial fraction was prepared by differential centrifugation and separated on an SDS/8 % polyacrylamide gel. The separated samples were transferred on to a nitrocellulose membrane and blotted with antibodies to LRPPRC and the 49 kDa subunit of complex I (CI49k). After washing to remove unbound primary antibodies, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG and developed with ECL solution. (A) LRPPRC content in fibroblast cell mitochondria. N, normal cells; H, heterozygous cells; LSFC, fibroblast cells from LSFC patients. (B) LRPPRC content of liver mitochondria. Lane 1, normal liver mitochondria; lanes 2–4, liver mitochondria from LSFC patients. Locations of LRPPRC and CI49K are indicated on the right side. Migration of standard proteins of designated molecular mass (MW) are marked on the left side. CI49K is a mitochondrial-specific protein. Histone H1 is a nuclear-specific protein.

**Figure 4** Western-blot analysis of LRPPRC content in nuclei

Nuclear fractions were prepared by removing cell membranes, organelles and cytoplasm in a low-salt/0.1 % NP40 lysis buffer followed by extraction in a high-salt extraction buffer. Samples from either normal or LSFC fibroblast cells were separated on an SDS/10 % polyacrylamide gel and transferred on to a nitrocellulose membrane. The membrane was probed with antibodies to LRPPRC, CI49K and histone H1 respectively. Upper panel: anti-LRPPRC and anti-CI49k blots. Lower panel: anti-histone H1 blot. Lane 1, normal mitochondrial fraction from cell line 4212; lanes 2 and 3, normal nuclear fraction from cell lines 4212 and 3838; lane 4, nuclear fraction from 4254 heterozygous cells; lanes 5–7, nuclear fraction from cell lines 3516, 7143 and 7240 from LSFC patients. CI49K is a mitochondrial-specific protein. Histone H1 is a nuclear-specific protein.

**Figure 5** \(^{35}\)S\(\)Methionine pulse-labelled human mitochondrial translation products

Normal or patient fibroblast cells were pulse-labelled with \(^{35}\)S\(\)methionine in the presence of 100 \(\mu\)g/ml emetine for 90 min. The mitochondrial fraction was prepared by differential centrifugation. Radiolabelled protein was determined by scintillation counter. Total labelled protein (10 000 c.p.m.) was loaded on each lane and separated on an SDS/polyacrylamide gel (10–20 % linear gradient). The separated samples were transferred on to a nitrocellulose membrane by electrophoresis and the membrane was exposed to an X-ray film. MTCO1, MTCO2 and MTCO3 denote subunits 1, 2 and 3 of COX respectively. MTND1, MTND2, MTND3, MTND4, MTND4L, MTND5 and MTND6 refer to subunits of the rotenone-sensitive NADH dehydrogenase. ATP6 and ATP7 are subunits 6 and 8 of \(\text{H}^+\)-ATPase. Migration of standard proteins is indicated on the left side. N, normal cells; H, heterozygous cells; LSFC, patient cells.

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cytochrome oxidase deficiency implies that LRPPRC as a PET 309 homologue is necessary for the normal expression of this enzyme in certain tissues [3]. The present study shows novel effects of this mutation on the observed biochemical phenotype. Northern-blot analysis suggests that mRNA levels encoding mutant LRPPRC protein are the same as controls, ruling out an effect on RNA stability. We showed, by immunofluorescence labelling, a set of images that suggest strongly that LRPPRC is in the mitochondria in control cells. However, in LSFC cells, LRPPRC seems to be diffusely situated in the cytosol and in most of the cells almost absent from the mitochondria. Moreover, the observed marked decrease in titre of LRPPRC in LSFC mitochondria, as observed by Western blotting, suggests that the protein is either not being produced in sufficient quantity, is not being efficiently imported or is being degraded after import. Experimental examination of import suggests that the precursor of LRPPRC is processed to the mature protein faster than the mutant A354V protein. A fraction of the LRPPRC protein seems to be associated disproportionately with the nucleus. Comparison with the level of the 49 kDa complex I subunit present in mitochondrial membranes suggests only a minor contamination of the nuclear fraction with mitochondria. However, LRPPRC is also reduced in the nuclei of LSFC patients. Since LRPPRC is a leucine-rich protein and a presumptive RNA-binding protein, it is possible that a proportion of the protein synthesized is directed towards the nucleus [9]. A further clue to the mechanism of action of the LRPPRC mutation is found in the specific decrease of the [35S]methionine signal for the synthesis of COX I and COX III subunits in an experiment to view specifically the synthesis of mitochondrially encoded proteins.

The lower levels of COX I and COX III mRNAs seen in LSFC patients suggest that LRPPRC has an mRNA-stabilizing function. Since LRPPRC is present in large amounts in heart and skeletal muscles, it is possible that the LSFC mutation does not completely remove the protective effect of the RNA-binding protein in these tissues. However, in tissues where expression is lower, the
mutation may have a more significant effect since the titre may be too low to give protection.

How do we reconcile the effect of the mutation (A354V) on the final COX activity with the postulated mechanism of action of LRPPRC? The LRPPRC gene has an mRNA expression profile typical of a mitochondrial protein, high in heart, skeletal muscles and kidney and low in brain and liver. On the other hand, when mutated, its impact on COX expression seems to be greatest in liver and brain, intermediate in skin fibroblasts and lowest in heart and kidney. This perhaps suggests that, wherever the expression of COX I is lowest, the assistance of LRPPRC is needed for stability. The alternative explanation for the differential effect of the mutation would be a scenario involving differences in the machinery or mechanism of COX translation or involving differences in the control of mitochondrial mRNA degradation in different tissues. At this time, there is no precedent for such differences, but such an explanation remains a possibility.

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