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The Leucine-rich Pentatricopeptide-Repeat Containing Protein Regulates Mitochondrial Transcription

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Abstract

Mitochondrial function depends upon the coordinated expression of the mitochondrial and nuclear genomes. Although the basal factors that carry out the process of mitochondrial transcription are known, the regulation of this process is incompletely understood. To further our understanding of mitochondrial gene regulation we identified proteins that bound to the previously described point of termination for the major mRNA-coding transcript H2. One was the leucine-rich pentatricopeptide-repeat containing protein (LRPPRC), which has been linked to the French-Canadian variant of Leigh syndrome. Cells with reduced expression of LRPPRC had a reduction in oxygen consumption. The expression of mitochondrial mRNA and tRNA was dependent upon LRPPRC levels, but reductions in LRPPRC did not affect the expression of mitochondrial rRNA. Reduction of LRPPRC levels interfered with mitochondrial transcription *in vitro* but did not affect the stability of mitochondrial mRNAs or alter the expression of nuclear genes responsible for mitochondrial transcription *in vivo*. These findings demonstrate the control of mitochondrial mRNA synthesis by a protein that has an established role in regulating nuclear transcription, and a link to mitochondrial disease.

The mitochondrial genome (mtDNA) encodes thirteen polypeptides that are critical constituents of the electron transport chain. However, the mitochondrion relies entirely upon nuclear-encoded genes for basic functions such as DNA replication and transcription. After translation, the mtDNA-encoded proteins function only through interactions with the larger cohort of imported, nuclear-encoded subunits. A variety of signals emanate from both the nuclear and mitochondrial compartments that help to integrate responses to a variety of bioenergetic stresses and maintain homeostasis (1,2). However, our understanding of the regulation of mitochondrial transcription, and its integration with nuclear transcription remains incomplete.

Mammalian mitochondrial genomes are small and organized with great efficiency. A short, partially triple-stranded region (the D-loop) contains protein-binding motifs that are implicated in mitochondrial DNA replication and transcription. Three primary transcripts are produced. The heavy-strand-encoded H1 transcript principally produces rRNA. The light strand-encoded L and the heavy-strand encoded H2 transcripts are processed to yield all of the mitochondrial mRNAs and the majority of the tRNAs (3). H2 is transcribed in the same

direction as H1 from a position that is slightly downstream from the H1 initiation point (4). In synthesizing the primary H2 transcript, the polymerase processes across nearly the entire span of the mtDNA and terminates only a few hundred nucleotides upstream of the initiation point (5). The primary transcript extends considerably beyond the final encoded gene (TrnP), but the purpose of this 3' extension is unclear.

Balancing the production of rRNA from H1 with the synthesis of mitochondrial mRNAs from H2 and L may be an important mechanism for regulating mitochondrial metabolism. The rRNA-containing H1 transcript is produced in vast excess to H2 *in vivo* (6). The mitochondrial transcription termination factor (MTERF) regulates this ratio, enhancing the transcription of H1 in preference to H2. MTERF does this by creating a physical linkage between the H1 initiator and terminator that is believed to allow the recycling of active transcription complexes (4).

The production of nuclear and the mitochondria-encoded subunits of the electron transport complexes must also remain in balance. Nuclear transcription factors, including NRF-1 and PGC1- α , play an important role in this process by driving the synthesis of both the mitochondrial transcriptional machinery and nuclear-encoded subunits of mitochondrial complexes (7,8,9). The MTERF-family member MTERF3, by contrast, acts within the mitochondria to repress heavy-strand transcription (10). Loss of MTERF3 causes severe respiratory deficiency in a mouse model, providing convincing evidence of the importance of transcriptional regulation (10). To further understand the regulation of mitochondrial transcription, we sought to identify additional regulatory proteins that bind within the mitochondrial d-loop to enhance the synthesis of mitochondrial mRNA.

MATERIALS and METHODS

Cell culture and transfection

HeLa cells were grown in high-glucose DMEM supplemented with 10% FBS (Gibco). siRNA were transfected using Oligofectamine (Invitrogen) according to the manufacturer's protocol. siRNA (Ambion) with the sequence GGUGCCAGCAAGAUUCUUGtt was used routinely for the silencing of LRPPRC but similar results were obtained with two siRNA sequences targeted to distinct regions of the gene. The reduction of LRPPRC was confirmed by Western blotting (Santa Cruz).

Gel shift assays

Oligonucleotide probes with the mitochondrial DNA sequence 228-258 (all sequence numbering uses the revised Cambridge reference sequence – accession AC_000021) were labeled by fill-in of 5' overhangs with 1U Klenow and 20 μ Ci α P³²-CTP. For binding reactions, 20,000 c.p.m. probe was combined with 15 μ g mitochondrial extract in the presence of 1 μ g salmon sperm DNA and 6 μ g bovine serum albumin. The complexes were incubated for 30 minutes at 37°C and resolved on a 4% acrylamide/0.5X TBE gel. Super shift analysis was performed using rabbit pre-immune serum or serum from rabbits immunized with LRPPRC after blocking the sera by incubation with unlabeled DNA. Competitor DNA sequences used included ggacataatTaACaATaAtgaatgtctgcac (“reversed”) and ggacataTAaaACTACTttgaatgtctgca (“scrambled”); bases mutated from the original 228-258 sequence are capitalized.

Transcription competent mitochondrial extracts

Mitochondrial extract preparation and *in vitro* transcription was performed as described (11), with several minor changes. Cell lysates were separated in 70mM sucrose, 210mM mannitol, 2mM HEPES pH 7.6, 0.5mM Na₂HPO₄. The mitochondria were disrupted by

resuspension in lysis buffer (20mM HEPES, 650mM KCl, 10mM MgCl₂, 1mM EDTA, 0.5% Tween-20) supplemented with protease inhibitors and membranes were removed by centrifugation at 100,000g for 30 minutes. Protein concentration was determined using a Bradford assay.

Purification and identification of D-loop binding proteins

Proteins binding to the 228-258 sequence were identified by affinity purification using sequential heparin-agarose, DNA-sepharose chromatography (12). Mitochondrial extracts were dialyzed against 20mM HEPES (pH7.4), 10mM MgCl₂, 1mM EDTA, 0.5% Tween-20. The extracts were initially fractionated by heparin-agarose chromatography using a stepwise KCl gradient. The fraction eluting at 0.5M KCl retained strong binding to the 228-258 sequence. This was dialyzed and re-fractionated on a DNA-sepharose column containing multimers of the target sequence using a stepwise gradient of KCl (0.3M to 1.0M). The purification was again monitored by gel shift assays and fractions with binding were combined and resolved by SDS-PAGE.

Bands were digested within the gel after reduction and alkylation (see donatello.ucsf.edu/ingel.html). The peptide fragments were identified by nanobore LC-MS/MS performed on an Agilent LC 1100 system interfaced to an ABI QStarXL mass spectrometer. The separation was performed on a 150mm reversed phase C18 column (Vidac). The spray capillary was a PicoTip Silica tip emitter with a 15μm tip (New Objective) with a curtain-gas setting of 15 and an ion spray voltage of 2500V. Nitrogen was used as a collision gas at a setting of CAD=5 and data were acquired in information-dependent acquisition mode. The peptides used to identify LRPPRC were IQEENVIPR and LIASYCNVGDIEGASK. The identification score threshold was 28. The Mascot program was used to search the Swissprot database was used for protein identification (13).

Respirometry

Oxygen consumption was analyzed using an Oxygraph-2K (Oroboros, Austria) as previously described (14). Cells were transfected with siRNA for two days, collected by centrifugation after trypsinization and added in fresh media at 1.5×10⁶ cells/mL. Samples were run simultaneously and the data was analyzed using the DatLab4 program.

Real-time PCR and quantitative PCR

Primer sets were designed using Primer Express 3.0 (Applied Biosystems). Real-time PCR was performed using SYBR Green PCR Master Mix per manufacturer instructions on a 7300 Real Time PCR system (Applied Biosystems). For analysis, the results were normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) expression. qPCR was performed on isolated total DNA and compared the levels of the mitochondrial target *CCO1* with the nuclear *COX4i1*. Samples were run in triplicate and are representative of three independent transfections. *p* values were calculated using a paired, 2-tailed, t-test (Excel).

Mitochondrial transcription

Sequences 16554-477 (L_s) or 15974-670 (L_i) were cloned into pCR2.1 (Invitrogen). The transcription templates were cleaved and isolated from vector DNA prior to use. Mitochondrial extracts (5μg) were incubated with templates (1μg) in the presence of 400μM ATP, 200μM GTP and CTP, 10μM UTP, and 10μCi αP³²-UTP then incubated at 32°C for 30 minutes. After treatment with proteinase K (20μg - 45°C for 30 minutes) the RNA was isolated by two rounds of extraction and precipitation and the resulting RNA was resolved on a 7% sequencing gel. Quantitation was performed with a STORM imaging system (Amersham), and statistics were performed as described above.

RESULTS

Protein binding at the site of H2 termination

MTERF regulates the initiation of H1 transcription through an interaction with the termination site (4). To investigate whether proteins binding at other sites of mitochondrial termination could also alter transcript levels, we identified proteins associated with human mtDNA at the point homologous to the known H2 termination site in mouse (5). Previous studies in our laboratory using mouse liver extract detected several proteins binding to this region, but these were not characterized (15). Gel-shift assays were used to monitor binding to the human termination point (Figure 1A). The fractions with probe-binding activity were resolved. Two bands were selected for identification by tandem mass spectrometric analysis of tryptic fragments. The first was the leucine-rich pentatricopeptide-repeat motif-containing protein (LRPPRC). The second protein identified was ATAD3. Both LRPPRC and ATAD3 have been previously identified in studies of proteins binding to mtDNA and ATAD3 is highly enriched in mitochondrial nucleoids and has a preference for binding to the mitochondrial D-loop (16,17,18).

Because the mass spectrometric identification of LRPPRC was based upon only two peptides we used gel-shift assays to further examine the association of LRPPRC with the H2 terminator (Figure 1B). Complexes formed by the interaction of mitochondrial extract and the terminator were disrupted by the addition of unlabeled competitor, as expected. The migration of the formed complexes was unaffected by the addition of non-specific IgG. The addition of a polyclonal antibody specific for LRPPRC resulted in the reduction of one of the two formed complexes and the appearance of a new band of high molecular weight, likely a ternary complex including LRPPRC and the antibody. To clarify the importance of the sequence in this interaction we used oligonucleotide competitors with mutations to interfere with the association of LRPPRC and the terminator. The terminator region for H1, which has little sequence similarity to the 228-258 region, was not competitive with the labeled H2 probe. We next used probes with sequences similar to the H2 terminator, and found that these were inefficient competitors with the labeled probe, suggesting that the interaction of LRPPRC with mtDNA was to some degree dependent upon sequence (Figure 1C).

siRNA-mediated reduction of LRPPRC interferes with mitochondrial respiration

We reduced LRPPRC levels by approximately 65% using siRNA transfection in HeLa cells (Figure 2A). Forty-eight hours after transfection, we evaluated respiration. Cells transfected with scrambled siRNA (control) had maximal respiration at baseline that was not altered by the addition of the uncoupling agent carbonyl cyanide-p-trifluoromethoxyphenylhydrazine (FCCP) (Figure 2B & 2C). By comparison, siLRPPRC cells had a reduction in both basal and FCCP-stimulated respiration when compared to control cells. Respiration in siLRPPRC was similar to controls in the presence of oligomycin and both lines were equivalently inhibited by the addition of the complex I inhibitor rotenone. These results suggest that normal expression of LRPPRC is important for the maintenance of electron transport.

Silencing of LRPPRC diminishes level of mitochondrial tRNA and mRNA

To evaluate the effect of LRPPRC depletion on mitochondrial transcription we performed real-time PCR analysis of mitochondrial mRNAs, rRNAs and tRNAs from siLRPPRC and control cells. There was a significant reduction in H2-encoded mRNAs such as *ND1*, *CCOI* and *CYTb* (Figure 3). We found a similar reduction in the levels of *ND6*, which is significant because it is the lone mRNA encoded on the light strand. Analysis of tRNA levels showed a reduction in levels of tRNAs derived from processing of H2 or L primary transcripts after LRPPRC silencing. In contrast, the two ribosomal RNAs were not affected

by LRPPRC silencing. The nuclear encoded *COX4i1* was also unaffected. These results agree with findings by other groups that have explored siRNA-mediated reduction in LRPPRC (19,20).

LRPPRC could increase mRNA levels by several mechanisms. LRPPRC is known to act as a nuclear co-activator through its interaction with PGC1- α and many PGC1- α dependent proteins function in metabolic and mitochondrial pathways (21). We examined the expression of mitochondrial transcription factor A (TFAM), TFB1M, TFB2M and the mitochondrial RNA polymerase (POLRMT) after silencing of LRPPRC by siRNA transfection and found that the levels of transcripts for genes involved in mitochondrial transcription were unchanged (Figure 3 - right). As a control, the nuclear encoded *CIDEA*, a previously known downstream target of LRPPRC, was reduced after LRPPRC silencing, showing that we had altered LRPPRC activity in the nuclear compartment.

Several direct mechanisms of LRPPRC action within the mitochondrion could also support mRNA levels. These include mtDNA maintenance, RNA stabilization and regulation of mitochondrial transcription. Depletion of the mitochondrial DNA would necessarily affect transcript levels. To exclude the possibility that LRPPRC silencing interferes with mitochondrial DNA maintenance we tested mitochondrial DNA levels using quantitative PCR and found that they were not significantly different from control cells (Figure 4A).

A second possibility is that LRPPRC may stabilize pre-existing mitochondrial mRNA. LRPPRC binds to poly-adenylated transcripts (22) and the assumed function of PPR-motifs is RNA binding. Furthermore, the work of other investigators has suggested that LRPPRC and SRA-stem loop interacting RNA binding protein form a complex containing mRNA (19). We tested the possibility that LRPPRC directly stabilizes mitochondrial mRNA by observing the decay of mitochondrial mRNA levels after treating cultured cells with actinomycin to block new RNA synthesis (Figure 4B). If LRPPRC contributed to RNA stability, we would expect that silencing LRPPRC would cause a more rapid decline in mRNA levels in combination with actinomycin treatment than would be seen with actinomycin treatment alone. To insure that the effect was relevant to both strands, ND1 and ND6 (products of the H2 and L strand respectively) were monitored. We found no increased instability in LRPPRC-silenced cells. Over the time course of actinomycin treatment, the levels of RNA in silenced and control cells converged and were not significantly different.

A final possibility is that LRPPRC influences mitochondrial transcription. This hypothesis was tested using an *in vitro* model of mitochondrial transcription. Mitochondrial protein extracts were prepared from control and siLRPPRC cells. These extracts were applied to two different linear mitochondrial DNA sequences that both contain an L-strand promoter driving run-off transcription (Figure 5). An analysis of the resulting labeled RNA showed that there was a reduction in transcription in cells with a reduced LRPPRC dosage. This suggests the importance of this protein in maintaining full transcriptional capacity of the L-strand promoter.

DISCUSSION

The coordination of mitochondrial transcription with nuclear gene expression is critical for proper organelle function (23). The nucleus must be able to regulate mitochondrial gene expression in response to environmental changes. This pattern of nucleus-to-mitochondria signaling is revealed by the changes in mitochondrial transcription seen after the forced expression or silencing of genes with nuclear transcriptional activity such as *NRF-1* and *PGC-1 α* (21,24). Physiologic changes including increased levels of thyroid hormone and exposure to cold can also provoke responses in mitochondrial transcription (25,26).

However, these are global effects upon transcription mediated by an alteration in the nuclear expression of proteins such as mitochondrial transcription factor A (TFAM). The ability to selectively modulate transcription or to balance the ratio of mRNA and rRNA may be an important cellular response. Pentatricopeptide-repeat motif containing proteins, a class that includes LRPPRC, have been previously implicated in the regulation of mitochondrial gene expression through mechanisms including translational and transcriptional control (27,28).

In this study we have observed that LRPPRC promotes the production of mitochondrial mRNA and several of the tRNA, without affecting rRNA. This finding has recently been reported by other groups studying the role of LRPPRC (19,20). The varied effects on differing mitochondrial RNAs is likely due to the different effects upon the three primary mitochondrial transcripts. Ribosomal rRNAs are created from the H1 transcript, whereas tRNAs and mRNAs are spliced out from the larger L and H2 transcripts (3).

Although it is possible that LRPPRC exerts its influence on mitochondrial transcription through its role as a nuclear co-activator, we have found that LRPPRC silencing does not alter the expression of mitochondrial transcription factors proteins such as TFAM. This observation was previously made in a different study of LRPPRC function (21,29). A dependence of some of mitochondrial transcription factors upon LRPPRC expression can be seen, but only in the context of forceful over expression of PGC1- α (21). We also saw no evidence that LRPPRC had a stabilizing interaction with pre-existing mitochondrial mRNA. Instead, LRPPRC seems to be required for maximal mitochondrial transcription.

Studies of proteins bound to the non-coding D-loop have identified a large cohort that includes LRPPRC (17,18). The diverse functions of this set of proteins suggest a close coordination of transcription, translation and DNA replication (30). We have found that LRPPRC binds to the mitochondrial D-loop at the point of H2 transcript termination. The molecular mechanism through which LRPPRC upregulates transcription has not been precisely determined in this study. There are possible similarities to the role of MTERF, which creates a physical bridge between the initiation and termination points of the H1 transcript (4). However, the effect of LRPPRC silencing on ND6, a product of L-strand transcription suggests that LRPPRC does not exclusively regulate H2. The proximity of all three mitochondrial promoters in the D-loop may allow proteins binding at a single point to regulate multiple transcripts.

We did not observe stabilization of the mitochondrial mRNAs ND1 and ND6 by LRPPRC. However, we do not exclude the possibility that LRPPRC may control mitochondrial metabolism by other means than transcriptional regulation. Indeed, Pet309, the yeast homologue of LRPPRC, is necessary for the translation of bound MTCOI mRNA (31). The concept of multi-purpose proteins within the mitochondrial matrix is not uncommon. The mitochondrial transcription factor B proteins are also rRNA methyltransferases (32). TFAM is a transcription factor but is also required for the maintenance of mitochondrial DNA (33,34). This functional efficiency may relate to the close coupling of mitochondrial functions by D-loop bound proteins or energetic barriers imposed by import of proteins into the mitochondrion.

It is interesting to consider our findings in the light of studies of French-Canadian Leigh Syndrome (LSFC - MIM 220111), which is caused by a missense mutation in *LRPPRC* (35). The A354V mutation impairs, but does not block, the entry of LRPPRC into the mitochondrion (36). Patients have reduced cytochrome oxidase activity and lower levels of both the mitochondria-encoded proteins MTCOI and MTCOIII and the corresponding mRNAs. The difference between the defect in cytochrome oxidase found in LSFC and the results we have obtained from a reduction of LRPPRC by siRNA may be due to specific

characteristics of the A354V mutation, which we did not replicate by siRNA-mediated reduction.

LRPPRC is also present in the nucleus and is active in regulating mitochondrial metabolism by control of nuclear gene expression. LRPPRC binds to PGC1- α and is required for PGC1- α mediated control of genes important for mitochondrial biogenesis and metabolism, as well as being required for brown fat differentiation (21,29). LRPPRC was also found to interact with mRNA export pathways (37). The identification of such diverse roles raises the intriguing possibility that LRPPRC may play an integrative role by co-regulating both mitochondrial and nuclear genes that encode the components of the electron transport chain. A review of the coordination of organellar and nuclear genome expression posited a prominent role for PPR-family proteins in this process (23). A recent study has also shown that PGC1- α is present within mitochondrial nucleoids (38), raising the possibility that LRPPRC activation in the mitochondrion may also depend upon interaction with PGC1- α . The exact mode of interaction between LRPPRC and the mitochondrial transcriptional machinery remains an important question.

The failure of mitochondrial protein synthesis leads to early-onset mitochondrial disease. Mutations in the mitochondrial DNA polymerase and associated proteins lead to a subsequent loss of mitochondrial DNA and constitute one of the more common mitochondrial syndromes of childhood (39). It has been suggested that a failure in mitochondrial transcription could lead to similar pathologies, but mutations in the known proteins required for mitochondrial transcription have not been found to be a primary cause of mitochondrial disease (40). Further studies using samples derived from LSFC patients may reveal that the impairment of the synthesis of mitochondrial mRNA is a pathogenic mechanism.

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Abbreviations

LRPPRC	leucine-rich pentatricopeptide-repeat motif containing protein
mtDNA	mitochondrial DNA
MTERF	mitochondrial transcription termination factor
GAPDH	glyceraldehyde phosphate dehydrogenase
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
CIDEA	Cell death-inducing DFFA-like effector A

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Figure 1. Identification of terminator bound proteins

A. Mitochondrial proteins were purified by sequential heparin-agarose (not shown) and DNA-sepharose chromatography. DNA-sepharose fractions were eluted with increasing salt concentration and binding was confirmed using gel-shift analysis. **B.** The binding of a probe with the distal terminator sequence by mitochondrial extracts was examined under various conditions. Binding was eliminated by the addition of an unlabelled competitor (50X cold). Super shifts were performed with either pre-immune serum or a polyclonal antibody to LRPPRC. A super shift was observed in the α -LRPPRC lanes with the transition of much of the slower migrating band (starred) to a new higher-mass complex. Free probe runs below the marked bands (not shown). **C.** The interaction of the terminator probe with mitochondrial extracts was competed using excess unlabeled DNA with and without mutations from the terminator sequence (rev. or scr. probes) or with the unrelated 3224-34256 sequence that binds MTERF1. Competitors effectively interfered with the interaction with the rapidly migrating band, but did not disrupt the slower moving band that interacts with the α -LRPPRC antibody (starred).

Figure 2. Respirometry in LRPPRC-depleted cells

A. Cells were transfected with siRNA against LRPPRC (siLRPPRC) or with an irrelevant siRNA (control) and grown for two days. LRPPRC protein levels were detected by Western blotting. Actin levels are provided below. B. Intact cells were analyzed using an Oroboros Oxygraph-2K with the samples run simultaneously in separate chambers. Both the oxygen concentration and slope are displayed. Inhibitor concentrations were oligomycin (4 μ g/ml), FCCP (2 μ M), rotenone (7.5 μ M) and antimycin (2.5 μ M). The tracings shown are representative of analyses of four independent transfections. C. Quantification and statistical analysis of oxygen consumption in control and LRPPRC silenced cells. Error bars indicate standard deviation (*) $p < 0.05$.

Figure 3. Gene expression in response to LRPPRC silencing

RNA was harvested from Control and siLRPPRC cells two days following siRNA transfection and the levels of mitochondrial transcripts were analyzed by real-time PCR. Values were normalized to GAPDH expression. Error bars represent the standard error of the mean. (*) $p < 0.05$.

Figure 4. LRPPRC silencing does not impair RNA stability or mtDNA maintenance

A. Quantitative PCR was used to compare the ratio of *CCO1* (mtDNA) and *CCO4-1* (nDNA) DNA in LRPPRC-silenced and control cell lines. **B.** The decay rate of mitochondrial mRNAs for ND1 and ND6 were evaluated after actinomycin treatment (5 μ g/mL). LRPPRC-silenced and control cells were cultured with actinomycin-D for the indicated times. RNA was extracted and ND1 and ND6 transcripts were analyzed by real-time PCR. (*) $p < 0.05$.

Figure 5. LRPPRC supports mRNA levels through a transcriptional mechanism

A. 5 μ g of mitochondrial extract from mock and LRPPRC-silenced cells were used in *in vitro* transcription reactions with linear L_s and L_1 templates, both containing the L-strand promoter. The resulting RNA was resolved on a 7% sequencing gel with a low-weight RNA ladder. The longer 3' extension of the L_1 template produces a runoff product 420 nucleotides longer than the L_s template. **B.** The intensities of run-off products obtained in three separate transcription reactions were quantified. Control and siLRPPRC values are significantly different ($p < 0.5$) for both L_s and L_1 -based transcription.