THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTY AND MOLECULAR BIOLOGY

PURIFICATION AND CHARACTERIZATION OF HUMAN LEUCINE-RICH PENTATRICOPEPTIDE REPEAT CONTAINING PROTEIN (LRPPRC) EXPRESSED IN *ESCHERICHIA COLI*

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ABSTRACT

French-Canadian Leigh Syndrome is caused by mutations in the gene encoding leucine rich pentatricopeptide repeat containing protein (LRPPRC). This disease is characterized by movement disorders (typical of energy deprivation disorders caused by mitochondrial dysfunction) the most common being dystonia. It may also present with seizures, loss of developmental skills, and culminate in kidney failure and heart problems. Consistent with a role in mitochondrial gene expression is the observation that the abundance of the mitochondrial mRNAs for two subunits of cytochrome c oxidase (COX1 and COX3) is reduced in patients with French-Canadian Leigh Syndrome. It is therefore hypothesized that mutations in LRPPRC disrupt the proteins ability to bind and stabilize these mitochondrial mRNAs for proper gene expression. In this thesis, we set out to establish an effective method for purification of LRPPRC. We also investigated the ability of LRPPRC to bind RNA and stimulate mitochondrial transcription in vitro. We succeeded in developing an effective and efficient purification process utilizing the pSUMO system. The purified LRPPRC protein was capable of binding RNA under conditions of low ionic strength. In addition, in vitro mitochondrial transcription assays revealed the potential for LRPPRC to stimulate transcription. Samples that contained purified LRPPRC protein had increased activity as compared with those that lacked LRPRRC. The development of this expression and purification procedure along with the corresponding biochemical assays can now be used to test the hypothesis that mutations in LRPPRC impair its normal function in the mitochondria that is required for proper gene expression.

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ACKNOWLEDGEMENTS

I have been blessed to have had the opportunity to join the Cameron Lab. In my time here, I have gone through immense growth, both as a scientist and as a person. I have learned so much from other members of the lab, especially from Dr. Craig Cameron and Dr. Jamie Arnold. The opportunities that have presented themselves to me through my work in the Cameron Lab have been tremendous. The chance to work collaboratively with Dr. Neal Sondheimer at the Children's Hospital in Philadelphia was an opportunity I never foresaw. These many men and women have been instrumental in shaping me and guiding me to an exciting future.

It has been an honor to be a part of the Penn State family. I have made so many friends in the past four years who have stood by me and supported me. The relationships I have cultivated with Dr. Hoover and Dr. Howell are ones that I will cherish for the rest of my life. They stood with me and talked me down when times were tough and have accepted me as one of their own.

My own family has been my greatest support during my tenure at Penn State. Through many hills and valleys, they have celebrated and wept with me. They are my greatest mentors and my greatest friends. These past four years have been challenging, and would have proven to be impossible without the love and support of my family, friends, professors, and colleagues.

Chapter 1

Introduction

The mitochondrion is a membrane-bound organelle present in eukaryotic cells. They are responsible for producing the majority of adenosine triphosphate (ATP) used by the cell. This is primarily accomplished through oxidative phosphorylation via the electron transport chain. In order for the electron transport chain to function, specific protein complexes are necessary to pass electrons further down the chain and generate a proton gradient. Several of these proteins are encoded by mitochondrial DNA (mtDNA) contained within the mitochondrion itself.

One of the essential proteins is cytochrome c oxidase (COX). COX represents the final transmembrane protein in the ETC, and is composed of four subunits surrounding an iron core. These subunits are assembled by a number of accessory proteins encoded by mtDNA, and mutations in these protein coding genes have led to a number of disorders including cardiomyopathy, leukodystrophy, and Leigh syndrome (1).

Leigh syndrome is a rare inherited neurometabolic disorder that affects the central nervous system with its onset being evident in infancy between the ages of three months and two years. There are a number of different variations, all of which are characterized by progressive and severe movement disorders and neurological symptoms. One such variant is French Canadian Leigh syndrome. The phenotype of the French Canadian variant is characterized by hypotonia (low muscle tone), distinct facial appearance, mental and physical developmental delay, ataxia, and serious episodes of illness that usually results from an infection. French Canadian Leigh syndrome occurs in approximately 1 out of every 40,000 children, but has an increased incidence of 1 in 2,000 amongst individuals of the Saguenay-Lac-Saint-Jean region of

Quebec. Unfortunately, few patients who suffer from this disease will not live beyond their first few years of life. The majority will suffer from fatal "crises" characterized by the build up of lactic acid due to the insufficient amount of ATP produced. Currently there are no treatment options.

French Canadian Leigh syndrome has been directly linked to the A354V mutation in the leucine-rich pentatricopeptide repeat containing protein (LRPPRC) (2). LRPPRC is a key accessory mitochondrial protein that has been shown to be involved in transcription and stabilization of mtRNA (3). PPR motifs have been observed as vital regulators of mitochondrial gene transcription and expression (4). Recent studies have linked LRPPRC to COX enzymes. In a 2004 study, it was observed that the A354V mutation in LRPPRC produced cells that were deficient in COX1 and COX3 mtRNA transcripts (5). This mutant protein, A354V-LRPPRC, was also shown to play a key role in altering mtRNA transcript stability leading to suppressed COX activity (6). Research conducted by Dr. Neal Sondheimer found that mutations in LRPPRC led to decreases in oxygen consumption (7). It was observed that expression of mitochondrial mRNA and tRNA was reduced with decreased expression of LRPPRC, but indicated that there was no change in mitochondrial rRNA (7). It has been hypothesized that LRPPRC binds and stabilizes COX1 and COX3 transcripts for proper post-transcriptional regulation of mtDNA gene expression through the interaction with ribonucleoprotein complexes (8). Mutations in LRPPRC have been suggested to eliminate the protein's ability to bind and stabilize mtRNA transcripts (5).

We set out to develop an efficient expression and purification procedure of LRPPRC from *E. coli.* This protein could then be used to study the hypothesis that LRPPRC functions in mRNA stability and mitochondrial transcription, and that the mutant form directly interferes with these functions. In this thesis, we devised a process that produces active and soluble LRPPRC through the utilization of the pSUMO system. This purified protein was then used to determine LRPPRC's ability to bind to RNA and to stimulate mitochondrial transcription in vitro. This

thesis has facilitated the means for further research into the characterization of LRPPRC. By using the tools developed here, it may now be possible to understand how mutations in LRPPRC play a role in regulating COXI and COXIII production, which will hopefully provide keys to developing an effective treatment plan for French Canadian Leigh syndrome.

Chapter 2

Materials and Methods

Materials – DNA oligonucleotides were from Integrated DNA Technologies, Inc; HerculaseII Fusion DNA polymerase was from Stratagene; Restriction enzymes were from New England Biolabs, Inc; Difco-NZCYM was from BD Biosciences; EDTA-free protease tablets were from Roche; Bradford reagent was from Bio-Rad; 3'-fluorescein-labeled rA15 (FL-rA_{15}) and rA20 (FL-rA_{20}) were from Dharmacon, Inc.; Ultrapure NTP solutions were from GE Healthcare; [α-^{32}P]UTP was from MP Biomedical. All other reagents were of the highest grade available from Sigma, Fisher, or VWR.

Construction of pSUMO-LRPPRC Bacterial Expression Plasmid – The gene encoding LRPPRC was amplified using a human cDNA clone (kind gift from Neal Sondheimer; ATCC# 9125880, IMAGE Clone ID# 4827456) as a template, oligonucleotides 1 and 2 (**Table 1**) and HerculaseII Fusion DNA polymerase. The PCR product was then cloned into pSUMO using BsmBI and NotI sites. The final construct, pSUMO-LRPPRC, was confirmed by sequencing at The Pennsylvania State University Nucleic Acid Sequencing Facility.

Expression of LRPPRC in *E. coli* by Auto-Induction – Rosetta (DE3) competent cells were transformed with the pSUMO-LRPPRC expression plasmid for protein expression. The transformed cells were plated onto NZCYM-agar plates supplemented with kanamycin (25 µg/mL) (K25), chloramphenicol (20 µg/mL) (C20) and dextrose (0.4%) and grown at 30 °C overnight. Rosetta(DE3) cells containing the pSUMO-LRPPRC expression plasmid were used to inoculate 100 mL of K25, C20-supplemented media (NZCYM) and grown at 37 °C to an OD_{600} = 1.0. This culture was then used to inoculate 500 mL of K75, C60-supplemented ZYP-5052 auto-

induction media [Studier PEP] to an OD_{600} = 0.025. The cells were grown at 37 °C to an OD_{600} of 1.0, cooled to 25 °C and then grown for an additional 16 - 20 h. The cells were analyzed by SDS-PAGE to confirm expression of the SUMO-LRPPRC fusion protein. Cells were then harvested by centrifugation at 6,000 x g for 10 min. The cell pellet was washed once with 200 mL of $T_{10}E_1$ (10 mM Tris, 1 mM EDTA, pH 8.0), centrifuged again at 6,000 x g for 10 min and the cell paste weighed. The cell pellet was then frozen and stored at -80 °C.

Purification of LRPPRC – Frozen cell pellets were thawed and suspended in lysis buffer (100 mM potassium phosphate at pH 8.0, 500 mM NaCl, 20% glycerol, 10 mM BME, 2.4 µg/mL pepstatin A, 2.4 µg/ mL leupeptin) to a concentration of 5 mL/g of cell paste. One EDTA-free tablet was added per 10 g of cell pellet. The cell suspension was lysed by passing twice through a French press (SLM-AMINCO) at 20,000 psi. The lysate was then placed immediately on ice and phenylmethylsulfonyl fluoride (PMSF) and nonidet P-40 (NP-40) were added to final concentrations of 1 mM and 0.1% v/v respectively. Lysates were clarified by centrifugation for 30 min at 25,000 rpm (75,000 g) at 4 °C using a Beckman JA 30.50 rotor. The clarified lysate was loaded onto a Ni-NTA column at a flow rate of 0.5 mL/min. The column was then washed with 10 column volumes of Buffer A (100 mM potassium phosphate at pH 8.0, 20% glycerol, 10 mM BME) containing 500 mM NaCl, 0.1% NP-40 and 5 mM imidazole. The column was washed a second time with Buffer B containing 5 mM imidazole. The column was washed a third time with Buffer B containing 500 mM NaCl and 50 mM imidazole. After the third wash the column was eluted with Buffer B containing 500 mM NaCl and 500 mM imidazole. Fractions were collected and assayed for purity by SDS-PAGE. Fractions with the highest purity were pooled, Ulp1 SUMO protease was added to 1 µg/ mg of SUMO-fusion protein and dialyzed overnight with Buffer B (50 mM HEPES pH 7.5, 20% glycerol, 10 mM BME) containing 500 mM NaCl. After dialysis and cleavage by the SUMO protease, the cleaved protein was passed over a HisPur Cobalt resin (Thermo Scientific) equilibrated with Buffer B

containing 500 mM NaCl to remove SUMO and Ulp1. The pass-thru was collected and diluted in Buffer B to final concentration of 50 mM NaCl. The diluted sample was loaded onto a phosphocellulose column equilibrated with buffer B containing 50 mM NaCl. The column was washed with Buffer B containing 200 mM NaCl. LRPPRC was then eluted with a linear gradient (6 column volumes) from 200 to 2,000 mM NaCl. Fractions were collected and assayed for purity by SDS-PAGE. Fractions with the highest purity were pooled, the protein concentration determined by Bradford assay, aliquoted and frozen at -80 °C until use.

Fluorescence Polarization RNA Binding Assays – Experiments were performed using a Beacon fluorescence polarization system. Assays were performed by mixing increasing concentrations of LRPPRC in a solution containing 10 mM HEPES at pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, either 10 mM or 100 mM NaCl, and 0.1 nM 3'-fluorescein-labeled RNA (FL-rA15 or FL-rA20). To determine the apparent dissociation constant ($K_{d,app}$), millipolarization (mP) was plotted as a function of LRPPRC concentration and the data were fit to a hyperbola **(Eq. 1)**:

Eq. 1: $$mP = (mP_{max} * [LRPPRC]/K_{d,app} + [LRPPRC]) + mP_0$$

where mP is the polarization measured, mP_{max} is the maximum polarization, mP_0 is the initial polarization in the absence of LRPPRC, and $K_{d,app}$ is the apparent dissociation constant.

In Vitro **Mitochondrial Transcription Filter Binding Assay** – Reactions were performed essentially as described in [JBC 2009 Lodeiro], however products were analyzed by the DE81 filter binding method. Briefly, reactions contained 1X reaction buffer (10 mM HEPES at pH 7.5, 20 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 0.1 µg/µL BSA), 4 nM linearized pUC18-LSP-HSP1 plasmid DNA, 1X NTP mix (400 µM ATP, 150 µM CTP, 150 µM GTP, 10 µM UTP, 0.2 µCi/µL [α-^{32}P]UTP), 100 nM TFAM, 20 nM TFB2, 20 nM POLRMT and LRPPRC (0 - 100 nM) (final concentrations). Proteins were diluted immediately prior to use in 10 mM HEPES, pH 7.5, 1 mM DTT and 20% glycerol. Reactions were performed by incubating linearized plasmid DNA in reaction buffer at 32 ^{o}C for 5 min and then adding in the following

order: TFAM, TFB2, LRPPRC and POLRMT. Between each addition of protein to the reaction there was an incubation time of 1 minute. After addition of POLRMT, the reaction was allowed to incubate at 32 ^{o}C for 5 min and then initiated by addition of NTP mix. The reaction was allowed to proceed for various amounts of time and then quenched by addition of 50 mM EDTA. For product analysis, 10 μL of the quenched reaction was spotted onto DE81 filter paper discs and dried completely. The discs were washed 3 times for 5 -10 min in 250 mL of 5% dibasic sodium phosphate and rinsed in absolute ethanol. Bound radioactivity was quantitated by liquid scintillation counting in 5 mL of Ecoscint scintillation fluid (National Diagnostics).

Data Analysis - Data were fit by nonlinear regression using the program KaleidaGraph (Synergy Software, Reading, PA). Standard deviation was determined based upon the difference between the data point and the curve fit line. All experiments shown are representative, single experiments.

Chapter 3

Results

Construction of pSUMO-LRPPRC Bacterial Expression Plasmid – Previous studies by the Cameron Lab have utilized the pET-Ubiquitin system for production of recombinant proteins in *E. coli* (9, 10). This system allows for the expression of ubiquin-fusion proteins that can be co- and/or post-translationally processed by a ubiquitin-specific carboxy-terminal protease. The advantages of this system are that it produces a high level of expressed and soluble protein and allows for the production of full-length mature proteins that contain the authentic amino-terminus.

Subsequent modifications to this system have allowed for the production of recombinant proteins that contain either an amino or carboxy terminal hexa-histidine tag that can be purified using affinity chromatography resins (11). However, one disadvantage of this modified system is that the final purified protein contains an external tag that could complicate further biochemical analysis. We therefore utilized a similar system that allows for the production of a fusion protein that can be purified by affinity resins but can be subsequently processed in vitro after purification to produce a full-length, mature protein with the absence of any tags.

This system is called the pSUMO-fusion system. It allows us to produce a SUMO-fusion protein that contains an N-terminal hexa-histidine tag on the amino terminus of SUMO. This protein can be purified using affinity chromatography and subsequently processed by a SUMO-protease, Ulp1.

In order to successfully amplify the gene encoding LRPPRC, Herculase PCR was utilized. We engineered LRPPRC to lack the mitochondrial targeting sequence, which is

composed of the first 59 amino acids. The oligos utilized for PCR are shown in **Table 1**. The final PCR product was engineered to possess both necessary restriction sites (BsmBI and NotI) for cloning into the pSUMO vector. This product was then purified via agarose gel electrophoresis purification, digested with restriction enzymes, and subsequently purified.

Ligation of the digested pSUMO vector and LRPPRC PCR product was performed and then transformed into DH5α cells. The final expression plasmid was isolated by Mini/Midi DNA prep purification, verified by restriction mapping and sequenced **(Fig. 1)**.

Expression of LRPPRC as a SUMO-Fusion Protein in *E. coli* by Using the Auto-Induction System – To determine if SUMO-LRPPRC is capable of being expressed in *E. coli*, our bacterial expression plasmid was transformed into Rosetta (DE3) competent cells. These cells were selected because they are uniquely designed to enhance the expression of eukaryotic proteins that contain codons that are rarely used in *E. coli*. Colonies were harvested and used to inoculate 100 mL of K25, C20-supplemented NZCYM media as a starter culture. The cultures were grown at 37 °C to an OD_{600} of 1.0, and were then used to inoculate a 500 mL culture of K75, C60-supplemented ZYP-5052 auto-induction media as described in Materials and Methods. The auto-induction system allows for the production of recombinant proteins in *E. coli* at high-cell densities using specialized formulated media (12). Instead of inducing expression by IPTG when the T7 RNA polymerase gene is under control of the l*acUV5* promoter, *E. coli* first uses glucose as a carbon source to reach high-cell density. Once glucose is consumed, lactose is preferentially metabolized to allolactose, which then induces expression of the T7 RNA polymerase gene. This in turn induces expression of a given target gene that is under control of the T7/*lac* promoter. This system is especially advantageous in that protein synthesis is induced at high-cell density resulting in greater yield and during this phase chaperone proteins are uniquely activated to assist with proper folding and solubility.

Table 1
Oligonucleotides Used in this Study

Oligo No.	Oligo Name	Sequence
1	LRPPRC-Δ59-BsmBI-for	5'-GCGGGTACC*CGTCTC*AAggt**GCCATTGCTGCCAAAGAAAAAGAT**-3'
2	LRPPRC-NotI-rev	5'-GCGGGTACC*GCGGCCGC*ctatta**AGAAGAGTTTTCCCTCAATTTTCTTAG**-3'

The coding sequence for LRPPRC is shown in bold. Restriction sites (BsmBI in Oligo 1 and NotI in Oligo 2) for cloning are italicized. The coding sequence for SUMO in Oligo 1 and the two stop codons in Oligo 2 are lowercase.

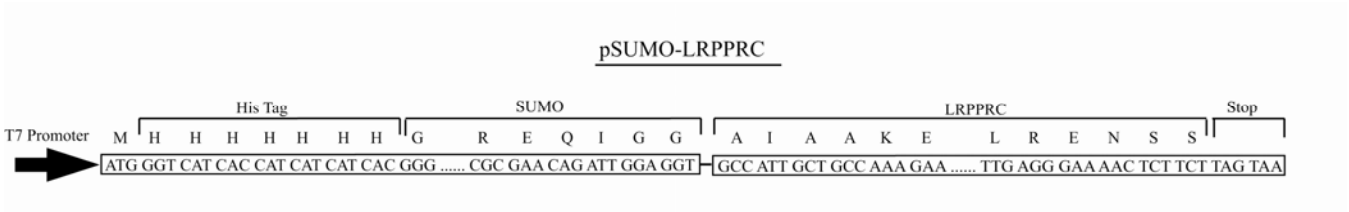


Figure 1. pSUMO-LRPPRC Bacterial Expression Plasmid. LRPPRC is cloned in frame with SUMO to produce a SUMO-fusion protein that can be purified using Ni-NTA chromatography and subsequently cleaved using Ulp1 to produce WT LRPPRC.

Both 1 mL uninduced and induced samples were collected at the appropriate times. The samples were run on an 8% SDS-PAGE gel and showed distinct induction of the SUMO-LRPPRC fusion protein located between 200 and 116 kDa markers with a theoretical molecular weight of 164 kDa **(Fig. 2)**. The induced cell culture was pelleted by centrifugation and harvested before being weighed and stored.

Purification of LRPPRC – The utility of this system allows for the production of soluble fusion protein that can be isolated via Ni-affinity chromatography. To test for soluble protein, harvested cells were lysed via French Press as described in Materials and Methods. The lysate was then clarified by centrifugation and analyzed by SDS-PAGE **(Fig. 3A)**. For appropriate controls, both uninduced and induced samples were loaded onto the gel along with the lysate and clarified lysate. This confirmed that SUMO-LRPPRC fusion protein was present in the clarified lysates and located in the soluble fraction after clarification.

The clarified lysate was loaded onto a Ni-NTA column and samples were collected for the initial load, the passthrough, washes, and elutions. This allowed for us to track the protein through the purification. SDS-PAGE gel analysis showed the presence of the SUMO-LRPPRC construct was minimal in the passthrough and washes, but was more prevalent in the final elutions **(Fig. 3B)**.

SUMO-LRPPRC was incubated with Ulp1 and allowed to cleave the SUMO-tag. This allowed for the production of a purified, mature LRPPRC protein without any tag that could be used in further assays. Analyzing samples of pre- and post-cleavage by SDS-PAGE showed a distinct shift in the bands **(Fig. 4)**. The post-cleavage product was passed over a Cobalt column to remove the SUMO-tag. The passes were collected and run on an 8% SDS-PAGE gel **(Fig. 4)**. The passes contained bands that migrated the same distance as the cleaved sample, indicating that the SUMO-tag had been cleaved.

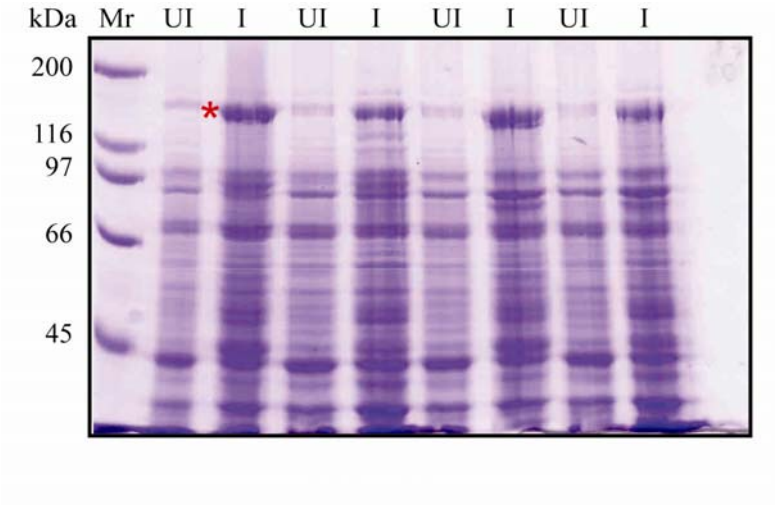


Figure 2. Expression and Purification of SUMO-LRPPRC. SDS-polyacrylamide (8%) gel of bacterial cell lysates before autoinduction (UI) and after 16 h of induction (I). The induced SUMO-LRPPRC fusion protein migrates between 200 and 116 kDa markers, with a theoretical MW of 164 kDa.

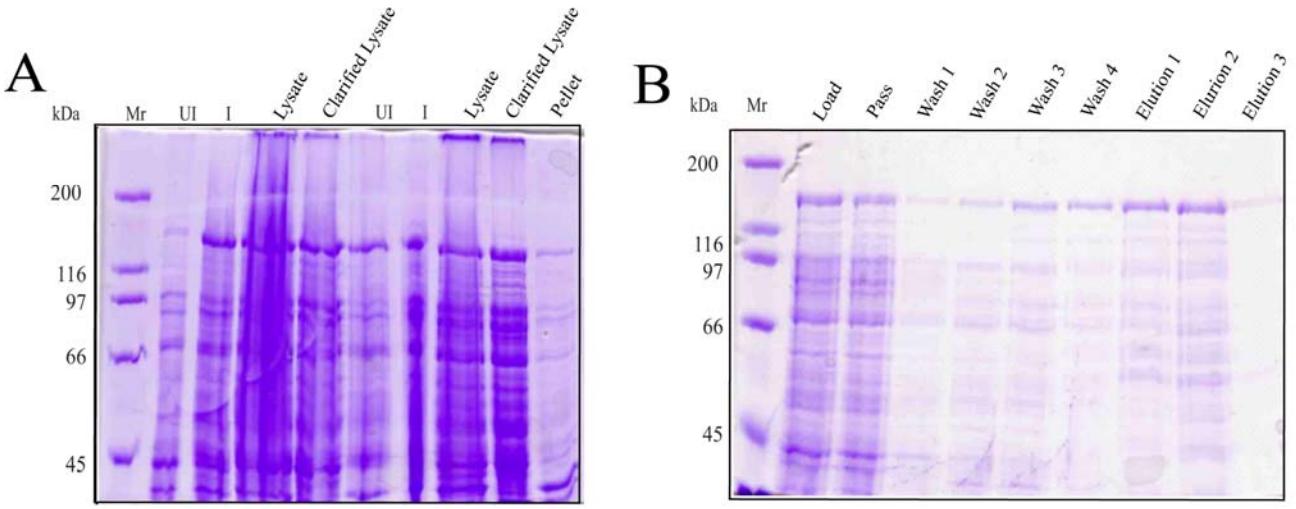


Figure 3. Purification of LRPPRC: Solubility and Ni-NTA Chromatography. Coomassie-stained SDS-polyacrylamide (8%) gel of the samples from each purification step. **(A)** Lane 1, molecular weight markers; lane 2, uninduced (UI) sample; lane 3, induced (I) sample; lane 4, lysate; lane 5, clarified lysate; lane 6, uninduced sample; lane 7, induced sample; lane 8, lysate; lane 9, clarified lysate; lane 10, cell pellet. **(B)** Lane 1, molecular weight markers; lane 2 Ni-NTA-agarose column load; lane 3, passthrough; lane 4, 5 mM imidizole wash; lane 5, second 5 mM imidizole wash; lane 6, 50 mM imidizole wash; lane 7, second 50 mM imidizole wash; lane 8, first elution fraction after addition of 500 mM imidizole; lane 9, second elution fraction after the addition of 500 mM imidizole; lane 10, third elution fraction after the addition of 500 mM imidizole. 20 μL samples from each fraction were boiled with one equivalent of 2x SDS sample buffer, and 10 μL was loaded on the gel.

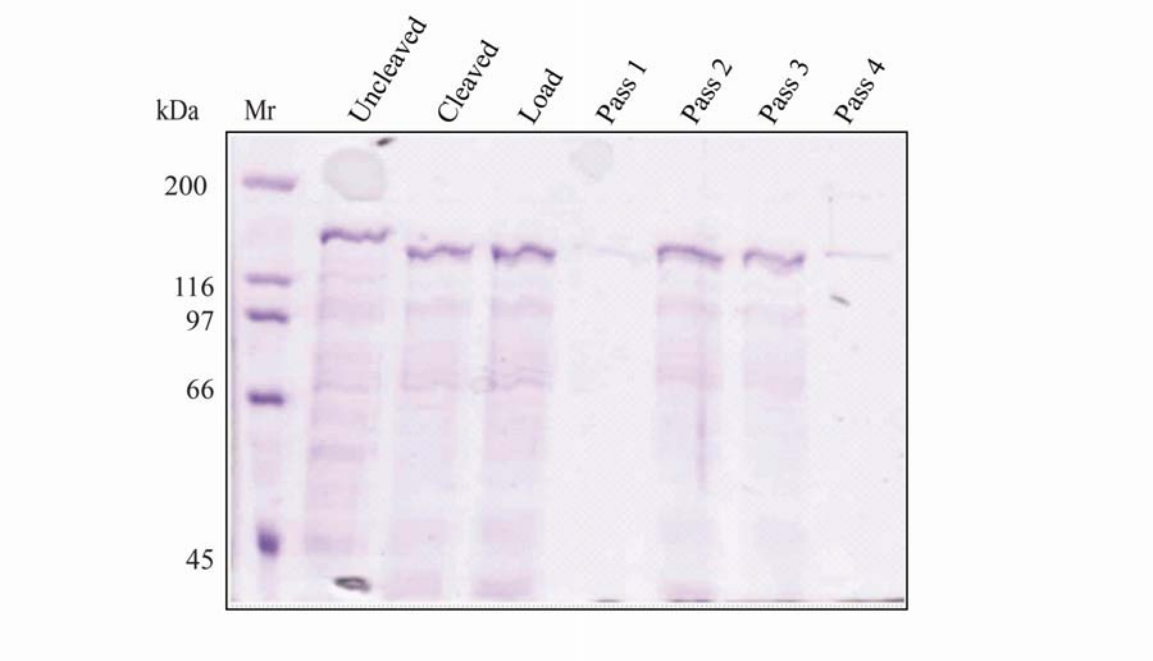


Figure 4. Purification of LRPPRC: SUMO Cleavage and Removal. Coomassie-stained SDS-polyacrylamide (8%) gel of the samples from each purification step. Lane 1, molecular weight markers; lane 2, Uncleaved SUMO-LRPPRC; lane 3, SUMO-LRPPRC cleaved via Ulp1; lane 4, Cobalt Load; lane 5, Cobalt passthrough 1; lane 6, Cobalt passthrough 2; lane 7, Cobalt passthrough 3; lane 8, Cobalt passthrough 4; lane. 20 μL samples were from each fraction were boiled with one equivalent of 2x SDS sample buffer, and 10 μL was loaded on the gel. As shown in lane 2, after addition of Ulp1, the SUMO-LRPPRC fusion protein was successfully cleaved to produce full length LRPPRC which has a MW of 152 kDa.

As a further purification step, the cleaved LRPPRC was passed over a Phosphocellulose (PC) column. It revealed the presence of 2 distinct elution peaks **(Fig. 5B)**. From observing the SDS-PAGE gels of the elution fragments, we determined that three pools of protein might be present and it was chosen to collect those pools and determine if they were alike **(Fig. 5A)**. Pooled fractions (Pool 1: 3-9, Pool 2: 20-22, Pool 3: 22-30) were analyzed by SDS-PAGE with 1 and 2.5 μg to estimate the total amount of protein present **(Fig. 6A)**. The final concentrations and volumes of purified LRPPRC were collected and quantified **(Fig. 6B**).

Purified LRPPRC Showed Binding to Fluorescently Labeled RNA *In Vitro* using a Fluorescence Polarization Assay – In order to determine if LRPPRC was capable of binding to RNA, we utilized a fluorescence polarization assay. Protein pools 1 and 2 were mixed with fluorescein-conjugated RNA and complex formation was detected as an increase in anisotropy of emitted light. LRPPRC (0 – 100 nM) was incubated with fluorescein-labeled rA15 or rA20. The change in anisotropy was plotted as a function of concentration of LRPPRC and the data was fit to an equation describing a hyperbola. It was determined that both LRPPRC pools 1 and 2 in the presence of 10 mM NaCl showed similar binding affinity to rA15 with a K_d value of 44 ± 2 nM (Pool 1) and 41 ± 2 nM (Pool 2) **(Fig. 7A)**. Pool 1 was selected to determine if there was any change in K_d if it was mixed with FL-rA20 **(Fig. 7B)**. The change in polarization was much less severe, and the K_d value was calculated to be 95 ± 2 nM.

In order to emulate the highly ionic conditions of the mitochondria, NaCl concentration was increased to 100 mM. Polarization (mP) was determined for both LRPPRC associated with FL-rA15 and FL-rA20 **(Fig. 8)**. At 100 mM NaCl, there was a significant decrease in mP for both rA15 and rA20 when compared to the mP calculated at 10 mM NaCl.

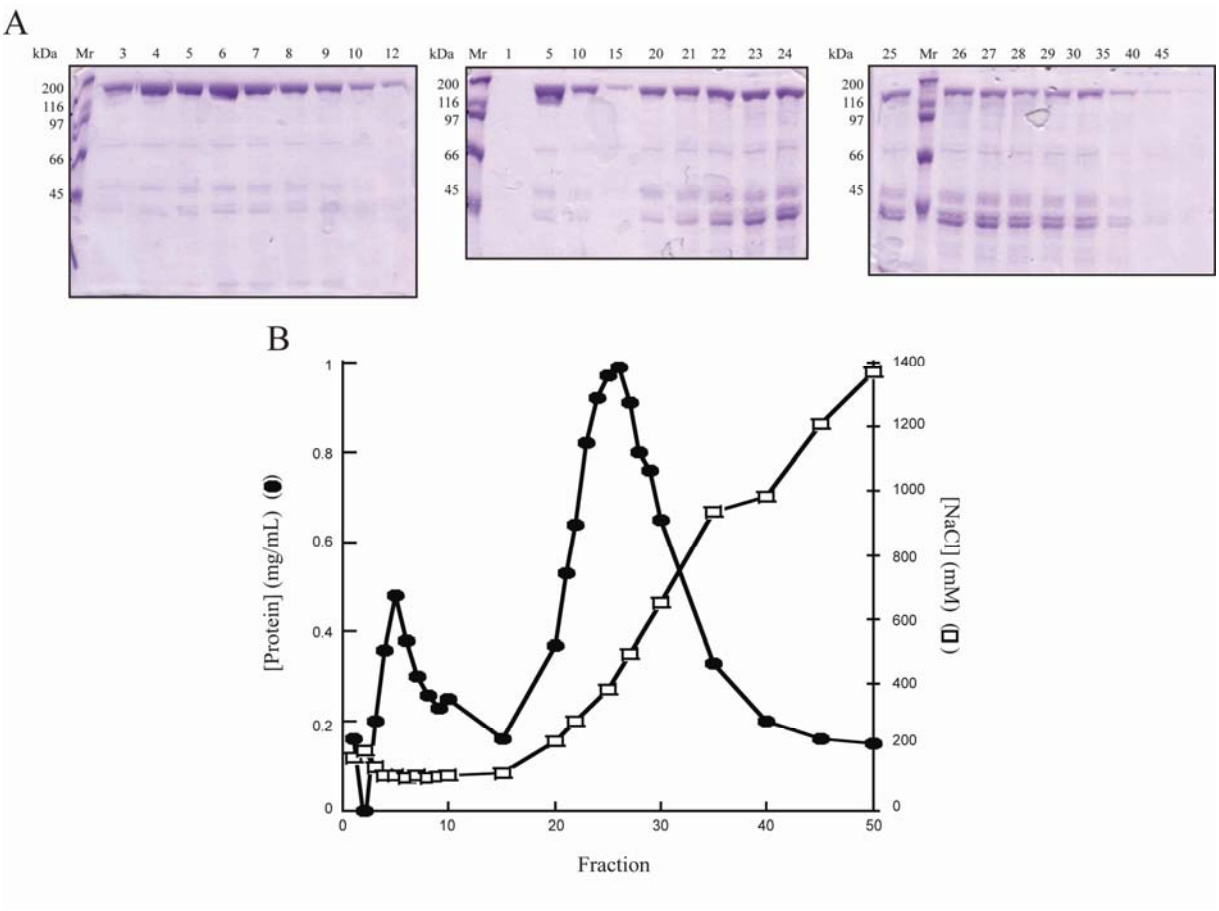
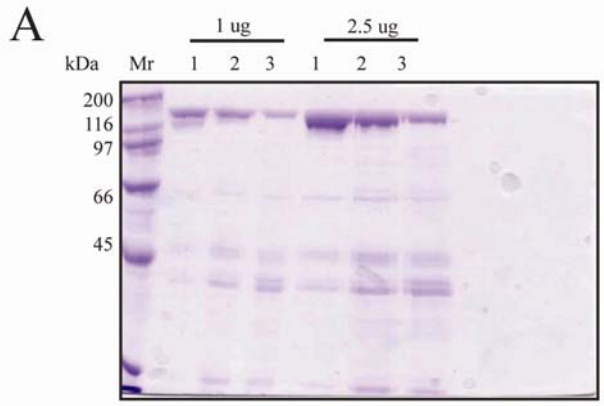


Figure 5. Phosphocellulose Chromatography: SDS-PAGE Analysis and Elution Profile. (A) Phosphocellulose elution fractions (3-12; 1, 5, 10, 15, 20-30, 35, 40, and 45) were resolved on an 8% SDS-PAGE gel. **(B)** Elution profile. LRPPRC was eluted from phosphocellulose column using a gradient from 100-1400 mM NaCl as described under Materials and Methods. Fractions 3-9, 20-21, and 22-30 were pooled uniquely and each pool was assayed for protein.

A



B

Pool	Volume (mL)	Concentration (mg/mL, μM)	Yield (mg)	NaCl (mM)
1	2.3	0.29, 1.9	0.67	109
2	0.6	0.34, 2.2	0.2	234
3	2.3	0.76, 5.0	1.75	479

Figure 6. Final Purified Pooled Fractions of LRPPRC: SDS-PAGE Analysis and Yield. (A) Coomassie-stained SDS-polyacrylamide (8%) gel of the final pooled Phosphocellulose elution fractions. 1 and 2.5 μg samples of each pool were loaded onto the gel and the intensity was used to estimate LRPPRC purity. **(B)** Final LRPPRC concentrations and volume of Phosphocellulose fraction pools determined by Bradford assay. Levels may be inflated due to lower molecular weight contaminants.

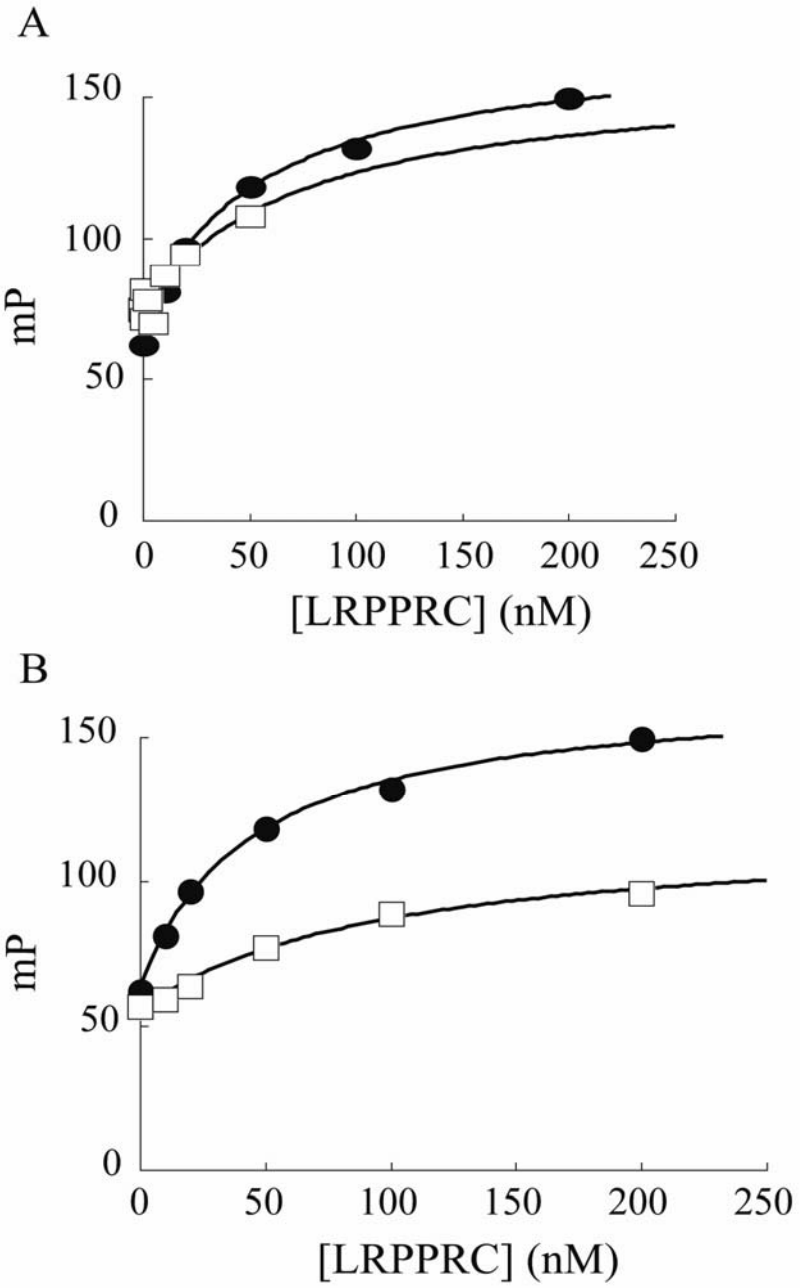


Figure 7. LRPPRC Binds to rA15 and rA20 Measured by Using a Fluorescence Polarization Assay. LRPPRC (0 – 200 nM) was incubated with a fluorescein-labeled rA15 and rA20 (0.1 nM) and the fluorescence polarization (mP) was measured via Beacon fluorescence polarization instrument. **(A)** LRPPRC Pools 1 (●) and 2 (▢) show similar binding affinity to fluorescently labeled rA15. **(B)** LRPPRC Pool 1 binds with greater affinity to FL-rA15 (●) than to FL-rA20 (▢). Change in fluorescence polarization was plotted as a function of LRPPRC concentration and fit to a hyperbola by using KaleidaGraph, yielding a Kd value of 41 ± 1.78 nM (Pool 2 rA15), 44 ± 1.97 nM (Pool 1 rA15) and 95 ± 2.00 nM (Pool 1 rA20).

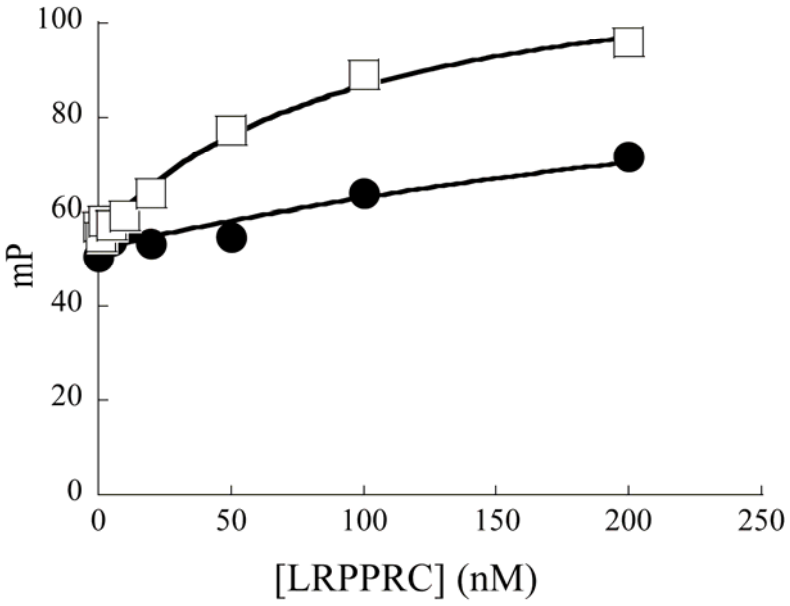


Figure 8. NaCl Inhibits LRPPRC Binding to FL-RNA. LRPPRC was incubated with fluorescein-labeled rA15 (0.1 nM) and the fluorescence polarization (mP) was measured via Beacon fluorescence polarization instrument. LRPPRC Pool 1 shows increasing mP at both 100 mM (●) and 10 mM (□) NaCl, however mP is significantly higher in 10 mM NaCl. Change in fluorescence polarization was plotted as a function of LRPPRC concentration and fit to a hyperbola by using KaleidaGraph, yielding a Kd value of 55 ± 1 nM (Pool 1 10 mM NaCl) and 52 ± 1 nM (Pool 1 100 mM NaCl)

LRPPRC Stimulates Mitochondrial Transcription *In Vitro* – Mitochondrial transcription assays were employed to determine if LRPPRC is capable of stimulating transcription. Previous studies have suggested that LRPPRC, in addition to binding and stabilizing interactions, plays a role in upregulating mitochondrial transcription. To explore this possibility, we utilized a mitochondrial transcription assay that was developed by Lodiero et al to test if the presence of LRPPRC can stimulate and/or increase transcription (11). Radioactively labeled [α-^{32}P]UTP was used to determine if incorporation had occurred. Samples were collected at distinct time points and quenched with EDTA before being spotted onto DE81 filter paper discs. The discs were dried and then washed in 5% dibasic sodium phosphate and rinsed in absolute ethanol. Final radioactivity counts were determined by using a scintillation counter. LRPPRC was added to one sample while buffer was added to the negative control. A significant increase in radioactive counts was noted in the samples containing LRPPRC compared to those lacking the protein **(Fig. 9)**.

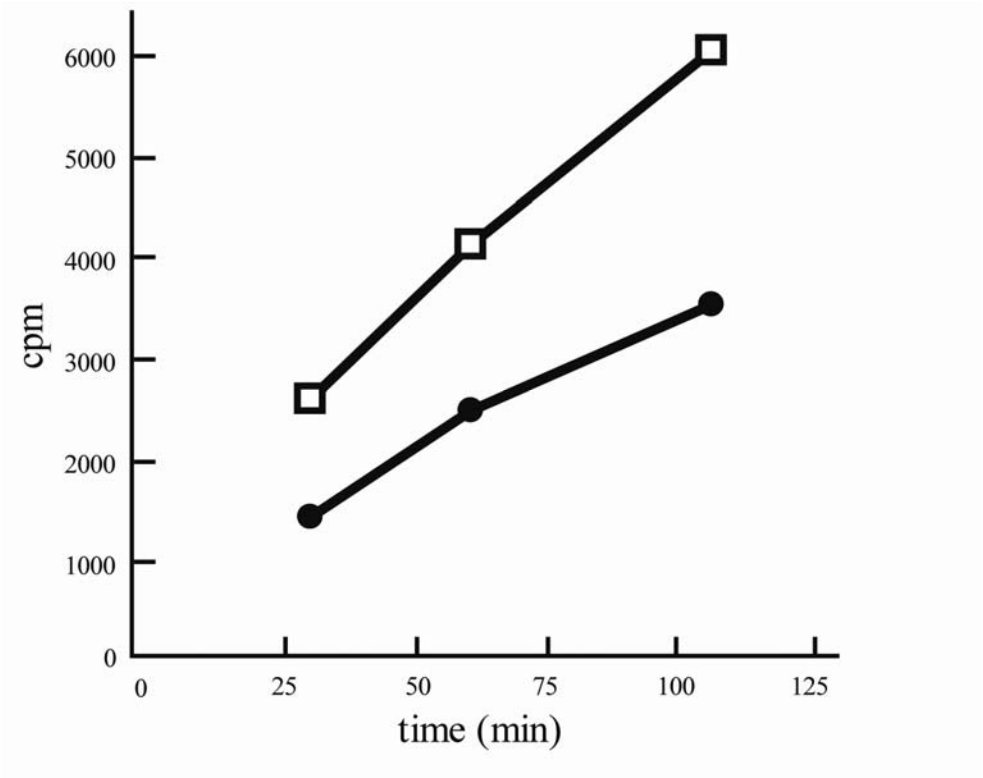


Figure 9. LRPPRC Stimulates Mitochondrial Transcription *In Vitro*. Transcription assay in the presence and absence of LRPPRC was performed as described in Materials and Methods. Samples were taken at 30 min, 60 min, 90 min, 105 min, and 120 min and quenched with 500 mM EDTA. 5 µL were then spotted on DE81 filter paper and radioactive counts were taken using a scintillation counter. In the presence of LRPPRC (❑) there is a significant increase of radioactive counts over time versus those that lack LRPPRC (●).

Chapter 4

Discussion

Development of an Efficient Expression and Purification Procedure for LRPPRC from *E. coli* – We describe a strategy for the efficient expression and purification of active human LRPPRC from E. coli. SDS-PAGE analysis confirmed both expression (**Fig. 2**) and solubility (**Fig. 3**) of SUMO-LRPPRC and also allowed for the effective tracking of this protein throughout the purification process. The presence of the hexa-histidine tag at the amino-terminus of the SUMO-fusion allowed for the use of Ni-NTA affinity chromatography for purification (**Fig. 4**). The SUMO-fusion was successfully processed by Ulp1 to release the authentic mature LRPPRC protein. Analysis of the Phosphocellulose (PC) Column fractions showed two distinct pools of protein that eluted from the column at increasing concentrations of NaCl, one pool at low NaCl and one pool at higher NaCl concentration **(Fig. 5)**. The basis for these distinct pools is not completely understood, however it is possible that these two pools may be a result of distinct biochemical behavior between these protein pools. For example, one pool of protein that binds to the PC column very tightly and one that interacts with lower affinity. Another possibility is the two peaks may be the result of proteolytically cleaved versions of LRPPRC that are forming a complex and binding tightly to the PC column preventing it from eluting at lower NaCl concentrations. The pool that eluted at higher NaCl concentration contained a significant amount of lower molecular weight contaminants when visualized by SDS-PAGE. Three pools were created from the two peaks (the second peak split into two pools). We chose to do this based upon the contaminants present in each sample **(Fig. 5).** The final concentration of these pools

could then be determined by SDS-PAGE and Bradford assays. Concentrations may be inflated due to lower molecular weight contaminants.

Utilizing the online tool ExPASy ProtParam, LRPPRC was determined to have a pI of 5.53. The PC column is designed to emulate the phosphodiester backbone of nucleic acids, and since LRPPRC binds to nucleic acids, it is likely that binding to the column is caused by binding motifs in the LRPPRC structure and not completely by electrostatic interactions.

LRPPRC Expressed in and Purified from *E. coli* Exhibits RNA Binding Activity – The purified LRPPRC was shown to exhibit RNA binding activity through use of fluorescence polarization assays. Experimental results indicated that the purified protein was capable of interacting with both fluorescently labeled rA15 and rA20. Pools 1 and 2 obtained from the PC elution showed similar trends in binding affinity and can therefore be used in exchange of each other in further RNA binding experiments **(Fig. 7A)**. Analysis of the graphical data showed that experiments utilizing rA15 and rA20 were reaching saturation. Interestingly, LRPPRC was shown to have a higher affinity to rA15 versus rA20, which contradicts what was hypothesized to be observed **(Fig. 7B)**. This however could be attributed to the quality of the fluorescently labeled rA20.

Experiments at biological levels of NaCl (100 mM) showed little to no increase in affinity as the concentration of LRPPRC was increased. However, at lower levels of NaCl (10 mM), there was a significant increase in affinity to fluorescently labeled RNA as concentration of LRPPRC increases **(Fig. 8)**. We can therefore suggest that the LRPPRC plays a distinct role in binding to mtRNA. However, more trials are necessary to explore LRPPRC's potential to bind mtRNA in biologically similar conditions.

LRPPRC Stimulates Mitochondrial Transcription Activity *In Vitro* – In order to further understand LRPPRC's role within the mitochondrial, we looked at its ability to influence transcription. Mitochondrial transcription assay showed that the purified LRPPRC was capable

of stimulating mitochondrial transcription in vitro **(Fig. 9)**. We clearly see a significant increase in incorporation of radioactive labeled UTP in samples that contained LRPPRC versus those that contained NaCl. The graphical analysis shows that the samples lacking LRPPRC were leveling off, while those that contained LRPPRC were still increasing. Further time points would be necessary to determine at what point we lose linearity. This is significant because it presents further understanding into how LRPPRC influences mitochondrial gene expression. As past research has shown, mutations in LRPPRC directly affect expression of COX1 and COX3 (5, 6, 7). These results present an alternative mechanism in which mutated LRPPRC fails to upregulate gene expression.

Chapter 5

Conclusions

In this thesis, we were able to show that WT LRPPRC can be successfully cloned, expressed and purified through the utilization of the pSUMO-fusion system. This provides the means for future experiments to quickly express and purify LRPPRC for further studies. The final protein was demonstrated to exhibit RNA binding activity *in vitro* and it is suggested that higher levels of NaCl may decrease LRPPRC's binding affinity to mtRNA. We also showed that LRPPRC has the ability to stimulate mitochondrial transcription *in vitro*. These results suggest that LRPPRC may not have a single function, but may act in both stabilizing mtRNA and upregulating mtRNA transcription.

The development of this purification procedure allows for future endeavors into the characterization of LRPPRC. Future experiments are necessary to further refine this purification technique. Utilizing anionic-exchange columns and gel filtration could effectively increase the final yield and purity of the final protein product. It is also necessary to definitively determine LRPPRC's binding affinity to mtRNA in differing monovalent salts and concentrations as well with different lengths of RNA. Further transcription assays can be utilized to characterize and define LRPPRC's role within the mitochondria. Even more pressing, research looking into site-specific mutations of LRPPRC would allow for an understanding of how these mutations influence the protein's ability to bind to RNA and/or stimulate mitochondrial transcription. Understanding these characteristics would allow us to describe the mechanism behind the phenotype of French Canadian Leigh syndrome, and may allow for potential therapeutic measures to alleviate the symptoms.

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Research Interests (usually for graduate applicants and prospective professors)

I have a great interest in infectious disease and epidemiology. Understanding how viruses and bacteria affect people, at both cellular and population levels, is an exciting area of research in this ever evolving world. The emergence of new infectious diseases (Ebola Virus, Marburg Virus, West Nile Virus, etc.) present new challenges to both physicians and researchers to provide prophylactic and post-exposure therapies. I hope to one day become a part of the dialogue and solution that sees these infections subdued or hopefully eradicated.