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Regulation of mitochondrial ribosomal protein S29 (MRPS29) expression by a 5'-upstream open reading frame

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Abstract

Mitochondrial ribosomal protein S29 (MRPS29) is a mitochondrial pro-apoptotic protein also known as death associated protein 3 (DAP3). Over-expression of MRPS29 has been reported to induce apoptosis in several different human cell lines while conferring resistance in glioma and *Ataxia telangiectasia* cells. These two contradictory reports led us to investigate the MRPS29-induced apoptosis further. *Cyber* searches of the EST databases revealed the presence of a splice variant of *MRPS29* mRNA containing an upstream open reading frame (uORF) at the 5' untranslated region (UTR). In this study, we confirmed the presence of this uORF using real time RT-PCR and investigated its role in MRPS29 expression.

Keywords

DAP3; uORF; Mitochondrial Translation; Apoptosis; Ribosome

1. Introduction

Mitochondria are powerhouses of cells providing over 90% of cellular energy and are also involved in many different functions including cell death in eukaryotic cells. They contain their own 16 kb circular DNA (mtDNA) and a unique translational machinery responsible for the synthesis of 13 mitochondrially encoded proteins. These proteins are all essential components of the respiratory chain complexes involved in the production of ATP by oxidative phosphorylation (Kurland, 1992; Lestienne, 1990; Wallace, 1992). Although the mitochondrial translational machinery has some similarities to that of bacteria, mammalian mitochondrial 55S ribosome differs from its bacterial counterpart in terms of size, protein composition, and rRNAs (Hamilton and O'Brien, 1974; Koc et al., 2001a; Koc et al., 2001b). It is estimated to have about 80 proteins and our current data suggest that about half of these proteins have homologs in bacterial ribosomes while the remainder represents new classes of ribosomal proteins (Koc et al., 2001a; Koc et al., 2001b). We have recently discovered the post-translational modifications of mitochondrial ribosomal proteins by phosphorylation and

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acetylation and their possible roles in regulation of mitochondrial translation (Miller et al., 2009; Miller et al., 2008; Yang, 2009).

There is growing evidence that suggest the involvement of mitochondrial ribosomal proteins in cancer, apoptosis and other metabolic diseases (Chen et al., 2009; Chintharlapalli et al., 2005; Kissil et al., 1999; Lyng et al., 2006; Miller et al., 2004; Miller et al., 2008; Yoo et al., 2005). For example, four different mitochondrial ribosomal proteins such as MRPS29, MRPS30, MRPL37, and MRPL41 have been reported to play a role in apoptosis (Chintharlapalli et al., 2005; Kissil et al., 1999; Koc et al., 2001c; Levshenkova et al., 2004; Mariani et al., 2001). We had identified two of these apoptotic proteins, MRPS29 and MRPS30 in our proteomics studies and mapped the critical phosphorylation sites found in MRPS29 by tandem mass spectrometry. Moreover, we determined the essential Ser and Thr residues need to be phosphorylated for the induction of apoptosis (Miller et al., 2008). In fact, phosphorylation of MRPS29 by Akt kinase and LKB1, a Ser/Thr kinase, was reported to be important for suppression of anoikis and pro-apoptotic function of MRPS29 in osteosarcoma cells (Miyazaki et al., 2004; Takeda et al., 2007). Other than the pro-apoptotic proteins of mitochondrial ribosomes, there are also several MRPs and their mRNAs which are expressed differentially such as MRPS23, MRPL11, and MRPL28 in tumor cells or tissues (Lyng et al., 2006; Robbins et al., 1995). Several different mechanisms are also shown to regulate the expression of mammalian mitochondrial ribosomal proteins MRPS12, MRPL11, and MRPL12 by different splice or uORF containing variants to support protein synthesis in different physiological conditions (Calvo et al., 2009; Mariottini et al., 1999). Therefore, it is possible that the changes in expression of MRPs essential for mitochondrial protein synthesis/function might influence the balance between apoptosis and tumor formation due to the Warburg effect in mitochondrial energy production.

One such well-known mitochondrial ribosomal protein is MRPS29, a GTP-binding protein found as part of the small subunit of the mammalian and yeast mitochondrial ribosomes (Denslow et al., 1991; Koc et al., 2001c; Saveanu et al., 2001). MRPS29 was initially identified as a pro-apoptotic protein in functional knock-out studies using anti-sense cDNAs (Kissil et al., 1995). Further studies have shown that MRPS29 is anapoptotic mediator of tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), and FAS-induced apoptosis by activating caspases such as caspase-8 and by mitochondrial fragmentation in mammalian cells (Kissil et al., 1999; Miyazaki and Reed, 2001; Miyazaki et al., 2004; Mukamel and Kimchi, 2004). Recent knock out studies also revealed that MRPS29 is an essential gene and its deletion is lethal in embryos (Kim et al., 2007). MRPS29 is known as a positive mediator of apoptosis; however, its over-expression in different cells lines and tumors such as thyroid and leukemia is not completely understood yet (Jacques et al., 2009; Yasumura et al., 2004; Bo et al., 2004). In addition, over-expression of MRPS29 conferred resistance to the induction of apoptosis in *Ataxia telangiectasia* (AT) cells treated by streptonigrin and ionizing radiation, and in invasive glioblastoma and glioma cells treated by camptothecin (Henning, 1993; Mariani et al., 2001). These two contradictory reports led us to investigate the regulation of MRPS29 expression further in human cell lines.

In this study, we are reporting the presence of different splice variants of *MRPS29* discovered through *cyber* screening of human ESTs. One of these splice variants contains an uORF in the 5'-UTR, which suggests that this uORF possibly blocks and decreases the expression of full length MRPS29. This could be a possible explanation for the regulation of this protein in different cell and cancer types.

2. Materials and methods

2.1. Cells and reagents

Jurkat and K562 cells were grown in RPMI-1640 media with 10% (v/v) fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C with 5% CO₂ in a humidified incubator. HeLa, CV-1, and HEK293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) under the same conditions as Jurkat and K562 cells, and cells were routinely subcultured in the semi-confluent state. RPMI-1640, DMEM, FCS, and penicillin/streptomycin were purchased from Mediatech, Inc. (Herndon, USA).

2.2. Generation of plasmid DNA constructs

cDNAs containing the human MRPS29 variants were purchased from ATCC (Manassas, USA). GenBank accession numbers for MRPS29 and uORF-MRPS29 are NM_004632 and NM_033657, respectively. The mammalian expression vector, pcDNA 3.1(+) (Invitrogen, Carlsbad, USA), was used to subclone various MRPS29 constructs for over-expression of MRPS29 in mammalian cells. PCR products for MRPS29 constructs were digested with KpnI and XhoI and subcloned into the KpnI/XhoI site of pcDNA 3.1(+) vector. Constructs were subcloned into mammalian expression vectors containing Kozak sequences either from the vector or native sequences from MRPS29 mRNAs. Especially, uORF-MRPS29 constructs contained native Kozak sequence GAGGGAaugG. The enhanced green fluorescent protein vector pEGFP-N1 (Clontech, Mountain View, USA) was used to determine the location of MRPS29. For the GFP constructs, the full length, partial MRPS29, and the uORF constructs were cloned into the XhoI/KpnI sites of pEGFP-N1 after digestion with appropriate restriction enzymes. The firefly luciferase vector pGL3-Control and the *Renilla* luciferase vectors pRL-TK and pRL-SV40 (Promega, Madison, USA) were utilized for luciferase reporter assays.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from various cell lines using Rneasy Mini Kit (QIAGEN, Valencia, USA) and cDNA was synthesized with ThermoScript™ RT-PCR system (Invitrogen). Primers used were as follows:

GAPDH:	5'-GTCTTCACCACCATGGAGAAGG-3' (Forward)
	5'-ATGAGGTCCACCACCCTGTTGC-3' (Reverse)
MRPS29:	5'-ATGATGCTGAAAGGAATAACAAGG-3' (Forward)
	5'-TGCAGAAGATCCCGACAATTTTCACCCA-3' (Reverse)
uORF-MRPS29:	5'-ATGGACCGACACGGGTATTGTACCGCTGA-3' (Forward)
	5'-TGCAGAAGATCCCGACAATTTTCACCCA-3' (Reverse)

2.4. Quantitative real-time PCR

Quantification of the *MRPS29* mRNA levels was performed by ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, USA) using 200 ng of total RNA extracted from each cell line. The housekeeping gene *cyclophilin A* was used as an internal control to normalize mRNA levels in different cell lines. For the quantification, the cycle threshold (Ct) values for *MRPS29*, *uORF-MRPS29*, and *cyclophilin A* mRNA were measured according to the previously described method (Livak and Schmittgen, 2001). The relative amounts of *MRPS29* mRNA were calculated as $2^{-\Delta\Delta C_t}$ where $\Delta\Delta C_t = [(C_t \text{ MRPS29 or uORF-MRPS29 level of cell line} - C_t \text{ Cyclophilin}) - (C_t \text{ MRPS29 or uORF-MRPS29 level of HeLa} - C_t \text{ Cyclophilin})]$. To determine the ratio of *MRPS29* to *uORF-MRPS29*, amplification efficiency was verified using the known copy number of *MRPS29* and *uORF-MRPS29* plasmids in PCR reactions. *MRPS29* and *uORF-MRPS29* amounts in each cell lines were calculated as relative folds of HeLa *MRPS29* and *uORF-*

MRPS29 levels. The following primer and probe sequences were used for real-time PCR designed by software supported by Applied Biosystems:

MRPS29: 5'-CCCCAGGATTTGGAGACTGTATT-3' (Forward)
 5'-GAATGTCTTCACCTGCATCACAA-3' (Reverse)
 5'-CCCCATGGCCTTCCTCCTCGC-3' (Probe)
 uORF-MRPS29: 5'-GGTCGCCTAGTCTGGAGAACTAGT-3' (Forward)
 5'-GGAGTCCCGCTCCTTTCC-3' (Reverse)
 5'-AGGGAATGGACCGACACGGGTATTGTAC-3' (Probe)

2.5. Cytotoxicity assays

Cell cytotoxicity was measured using WST-1 colorimetric cell proliferation assays (Roche, Basel, Switzerland) based on the cleavage of tetrazolium salt, MTS, by mitochondrial dehydrogenases in viable cells. Approximately 5×10^3 cells were seeded in 96-well plates without antibiotics. Transfections were performed after cells attached to the plates. For the transient transfection, the cells underwent a further incubation period (48 h) and then 10 μ l/well of WST-1 solution were added for cell viability assays. The absorbance was measured at 450 nm versus a 650 nm reference using a Microplate Reader (Molecular Devices, Sunnyvale USA). Results were expressed as mean $\pm$ SD and analyzed using the ANOVA's *t* test. Values of $\star P < 0.05$ were considered statistically significant.

2.6. Luciferase reporter assays

The pGL3-control vector (Promega, Madison USA) was utilized to subclone 5'-UTRs from *MRPS29* cDNAs for firefly luciferase assays. After amplification and digestion of 5'-UTR containing uORF of *MRPS29* with HindIII and NcoI, the PCR product was cloned upstream of the *luciferase* gene in the pGL3-Control vector. The primers 5'-AAA AAA AAG CTT TCT CAG GAC GGG CGC TTT GGA-3', 5'-AAA AAA CCA TGG CAT CCT TGC ACT CCT GGA-3' were utilized for the PCR amplification. The pRL-TK and pRL-SV40 vectors were used for *Renilla* luciferase constructs. For luciferase assays, approximately 5×10^4 of HeLa and CV-1 cells were seeded in a 24-well plate in antibiotic-free media. After attachment, cells were transfected with luciferase constructs using Lipofectamine 2000 (Invitrogen) and then cells were lysed in passive lysis buffer (Promega) at 48 h post-transfection. Firefly and *Renilla* luciferase signals were measured by Dual-luciferase reporter assay system (Promega) in a Junior LB 9509 luminometer (Berthold Technologies, Oak Ridge, USA). Results were expressed as mean $\pm$ SD and analyzed using the ANOVA's *t* test. Values of $\star P < 0.05$ were considered statistically significant.

2.7. Western blot analysis

Protein samples for Western blot analyses were prepared from cells lysed in a buffer containing 50 mM Tris-HCl pH 7.4, 150mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 0.1% SDS, and a protease inhibitor cocktail (Sigma, St. Louis, USA). After incubation of the whole cell lysates for 10 min on ice, soluble protein fractions were collected by centrifugation for 15 min at $14,000 \times g$ at 4°C. Protein concentrations were determined by BCA assays (Pierce, Rockford, USA), using BSA as a calibration standard. Approximately, 20 μ g of protein lysates were electrophoresed on a 10–16% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, Richmond, USA). Membranes were blocked for 2 h in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% (w/v) dry skim milk powder. Blotted membranes were washed with TBST (TBS containing 0.1% Tween-20) three times and incubated at 4°C with primary and secondary antibodies. The bound antibody signal was detected by chemiluminescence SuperSignal® West Pico kit (Pierce). Western blot analyses were performed with β -actin

(Abcam, Cambridge, USA), cleaved-PARP (Cell Signaling, Danvers, USA), His-Tag (MBL, Woburn, USA), MRPS29 and HSP60 (BD Biosciences, San Jose, USA) as primary antibodies.

2.8. Confocal microscopy

HeLa and CV-1 cells transfected with GFP fusion constructs were seeded in glass slides and stained with MitoTracker Red CMXRos (Molecular probes, Eugene, USA) and DraQ5 (Biostatus Limited, Leicestershire, UK) for mitochondrial and nuclear localization signals, respectively. Images were examined using a confocal microscope (Olympus Fluoview 300 Confocal Laser Scanning Microscope, Melville, USA).

3. Results

3.1. Presence of the uORF-MRPS29 in human cell lines

We discovered at least two different splice variants of *MRPS29* mRNA (NM_004632 and NM_033657) through human EST database searching. Partial sequence alignment for two of these *MRPS29* splice variants, one of which contained an uORF in 5'-UTR, was shown in Fig. 1A. Only about 30% of the ESTs available in the public databases contained the uORF in their 5'UTRs. Moreover, genomic DNA analysis by UCSC Genome browser (<http://genome.ucsc.edu/>) revealed an alternative splice junction in the intron region of the *MRPS29* gene. To confirm the presence of these uORF containing *MRPS29* mRNAs (*uORF-MRPS29*), RT-PCR analysis was performed using total RNA isolated from a monkey and several different human cell lines. Our results from the RT-PCR analysis demonstrated the presence of the *uORF-MRPS29* in several human cell lines, but not in the CV-1 (African green monkey kidney cells) (Fig. 1B). This observation was in agreement with the sequence databases as the uORF-containing ESTs were only found in human cell lines such as the Jurkat (human T cell leukemia) and HeLa (human cervical cancer cell) and tissues.

For quantitation of *MRPS29* and *uORF-MRPS29* expression, we performed real-time PCR analyses in HeLa, K562 (human chronic myelogenous leukaemia), Jurkat, and HEK293T (human embryonic kidney) cells. Changes in expression levels of *MRPS29* and *uORF-MRPS29* mRNAs in different cell lines were reported as fold of the mRNA levels in HeLa cell lines after normalizing the values against one of the housekeeping genes, *cyclophilin A* (Fig. 1C). Interestingly, expression of both mRNAs changed cell line to cell line; however, relative changes in both *MRPS29* and *uORF-MRPS29* levels were consistent in all cell lines (Fig. 1D). This observation coincides with the microarray data available for DAP3 in public gene expression databases (<http://wombat.gnf.org/index.html>) (Su et al., 2004). The ratio between the *MRPS29* and *uORF-MRPS29* mRNAs was calculated as the percentage of relative fold. For example, the total *MRPS29* level was 4.4 and the contribution from *uORF-MRPS29* was 1.1 in HeLa cells; therefore, percentages of *uORF-MRPS29* and *MRPS29* were calculated as 20.1% and 79.9%, respectively, in these cells (Fig. 1D).

Changes in expression of the *MRPS29* transcript in the real-time PCR analyses were also supported by variation in protein expression levels in different cell lines. As shown in Fig. 1E, the amount of *MRPS29* protein expressed in the same cell lines was also in agreement with the mRNA expression obtained from the real-time PCR analyses. Here, Western blot analysis performed with the HSP60 and β -Actin antibodies served as equal loading controls (Fig. 1E). To confirm the linearity of the signal intensity obtained with these antibodies, different amounts of proteins were separated on SDS-PAGE and probed with the same antibodies (Fig. S1).

3.2. Regulation of *MRPS29* translation by the 5'- uORF

To assess the role of the uORF found in the 5'-UTR of the *uORF-MRPS29* mRNA, luciferase reporter assays were performed using CV-1 and HeLa cells. In these assays, 5'-UTR of the

uORF-MRPS29 and an AUG→UUG mutant created at the AUG codon of the *uORF-MRPS29* constructs were cloned into the pGL3-control vector upstream of the luciferase. The pGL3-control vector containing the *luciferase* gene was used as a positive control due to continuous production of firefly luciferase. The schematic diagrams of different constructs used in luciferase assays are shown in Fig. 2A. Here, reduced expression of the luciferase was anticipated if the initiation codon of the uORF in the 5'UTR was recognized by ribosomes due to a frameshift generated in the coding sequence of *luciferase*. On the other hand, if the initiation codon in the uORF was not recognized by ribosomes, expression level of luciferase would be similar to its expression from the pGL3-control vector. Dual luciferase assays were performed in CV-1 cells transfected with firefly luciferase constructs and *Renilla* luciferase expressing vectors pRL-TK and pRL-SV40 to normalize and reduce differences in transfection efficiency and variation in these experiments. As expected, the expression of luciferase decreased significantly in cells transfected with the uORF containing constructs (Fig. 2B). This finding clearly demonstrated the recognition of the initiation codon in the uORF of the MRPS29 5'-UTR rather than the initiation codon for the luciferase expression by cytoplasmic ribosomes. The HSV-thymidine kinase promoter (pRL-TK) is a relatively weak promoter compared to the early SV40 enhancer/promoter (pRL-SV40). For this reason, the relative luciferase activity (RLU) level, which was normalized by pRL-TK, has shown relatively high luciferase activity compared to the RLU level in pRL-SV40. Mutation of the initiation codon in the uORF containing construct, however, reversed the effect of the uORF and the luciferase activity was similar to that of the pGL3-control vector in the same cell line (Fig. 2B). The luciferase assays were also repeated in HeLa cells and similar results were obtained (data not shown). Our findings suggest that the MRPS29 protein expression can be regulated by the uORF found in the 5'-UTR of the alternatively spliced *MRPS29* mRNA.

Above, we have verified ribosome recruitment at the first initiation codon in the 5'-UTR of *MRPS29* using the luciferase reporter assays (Fig. 2B). We have also investigated a possible blockage of the translation of the full length *MRPS29* in cells by transiently expressing uORF-MRPS29 to understand the role of uORF in down-regulation of MRPS29 protein expression. Especially in eukaryotes, optimal context sequence, also known as Kozak sequence, could play a significant role in ribosome recruiting and the binding of the initiation codon for translation to start (Kozak, 2005). In a Kozak sequence, nucleotides at positions -3 and +4 are important bases for translation initiation. For a good Kozak sequence, these nucleotides should be purine and guanine bases, respectively. If these nucleotides are absent in the sequence, ribosomes can bypass the start codon due to a poor context. The Kozak sequence for *uORF-MRPS29* (GAGGGA**aug**G) is in fact a better sequence compared to the sequence for *MRPS29* (GCAAG**Gaug**A). In this case, ribosomes would prefer to bind to the start codon in the *uORF-MRPS29* rather than the first AUG in the coding sequence of *MRPS29* for the initiation of translation, and therefore, the full length MRPS29 could not be translated efficiently in the presence of this uORF. Moreover, the uORF ends with two consecutive UGA stop codons that overlap with the two possible initiation codons in the coding sequence of MRPS29 (Fig. 1A). To confirm this possibility, MRPS29 constructs containing the C-terminal His-tag were cloned into mammalian expression vector, pcDNA 3.1 (+) (Fig. 3A). As seen in Fig. 3B, increased MRPS29 levels were observed in cells expressing His-tagged MRPS29 constructs. However, expression of the uORF-MRPS29 containing construct significantly reduced the expression level of His-tagged MRPS29 protein as probed with both anti-MRPS29 and anti-His-tag antibodies. Recombinant His-tagged MRPS29 protein purified from *Escherichia coli* (Rec-MRPS29) was used as a control for detection with anti-His-tag antibody. Membranes were also probed with anti-HSP60 antibodies to ensure that the equal amount of protein was loaded. Theoretically, His-tagged MRPS29 protein would not be detected if the first initiation codon in the uORF was the only initiation codon, AUG, recognized by ribosomes in cells transfected with uORF-MRPS29 constructs. Due to leaky scanning of initiation codons by ribosomes or alternative translation initiation site(s), the full length MRPS29 could still be expressed at much

lower levels in cells over-expressing uORF-MRPS29 (Fig. 3B). Therefore, our findings suggest that the presence of the uORF in the 5'-UTR of *MRPS29* translationally controls and reduces the expression of the full length protein by blocking the ribosome binding at the first AUG in the coding sequence of the *MRPS29* gene.

As shown in Fig. 1A, in the case of ribosome binding to the first AUG in uORF of *MRPS29*, translation could start from the uORF of *MRPS29*, which would synthesize a 23-amino acid (aa) residue peptide (approximately 2.4kDa). Attempts to detect expression of the 23 aa residue small peptide encoded from the uORF-MRPS29 were also made. For this purpose, a FLAG-tag was incorporated at the 5' end of the uORF used for the expression of MRPS29 with C-terminal His-Tag in pcDNA3.1(+) vector (Fig. 3A). To detect the expression of the FLAG-tagged small peptide, enzyme linked immunosorbent assays (ELISA) were performed using anti-FLAG antibody. Nevertheless, expression of the FLAG-tagged small peptide was not detected with ELISA (data not shown). To determine the localization of this peptide, we utilized GFP-tagging method described below.

3.3. Role of the uORF in cellular localization of MRPS29 in mitochondria

The MRPS29 constructs described above, with and without an uORF, were subcloned into a mammalian enhanced GFP vector, pEGFP-N1, to study the effect of uORF in translocation of MRPS29 into mitochondria using confocal microscopy. The GFP tag was fused at the C-terminal end of MRPS29 constructs to maintain the native mitochondrial localization signal peptide at the N-terminus of the protein. For the cellular localization analysis of gene products, HeLa cells were transiently transfected with various GFP fusion constructs, MRPS29-GFP, uORF-MRPS29-GFP, and uORF-GFP, possibly expressing MRPS29 and 23 residue long peptide from the uORF (Fig. 4). After transfection with each constructs, cells were stained with MitoTracker Red CMXRos and DRAQ5 to localize mitochondria and nuclei, respectively. Control cells containing the pEGFP-N1 vector were used to demonstrate non-specific and diffused localization of ubiquitously expressed GFP protein in cells (data not shown). Fig. 4A showed images obtained from localization of the MRPS29-GFP fusion protein localized in the mitochondria as the fluorescent signal from the fusion protein overlapped with the red signal from MitoTracker. Even though mitochondrial localization of MRPS29 was clear in some cells, GFP-tagged MRPS29 protein was not expressed all possibly due to low transfection efficiency in HeLa cells (Fig. 4A). Next, we examined the effect of the uORF in cellular localization of MRPS29 and observed an overall decrease in the GFP signal in cells transfected with uORF-MRPS29-GFP (Fig. 4B). Here, some of the GFP-fusion protein(s) of the cells transfected with the uORF-MRPS29-GFP constructs appeared to express that was translocated into mitochondria (indicated by arrow heads), other cells ubiquitously expressed the GFP fusion protein giving a pattern similar to that obtained from the pEGFP-N1 control cells (Fig. 4B). This observation could be because of the impaired expression of the *MRPS29* coding region in cells transfected with the *uORF-MRPS29*, as described earlier (Fig. 1A). However, after recognition of the initiation codon in the uORF, ribosomes may continue to scan for additional in frame codons and translate N-terminally truncated MRPS29 proteins with the GFP tags by cap-independent manner or due to leaky scanning of initiation codons. To test this possibility, variety of MRPS29 constructs, including four of the in frame AUG codons, were cloned into mammalian enhanced GFP vector, pEGFP-N1. Each constructs was transfected into CV-1 cells to follow their localization in the cell (Fig. S2). Surprisingly, some of the N-terminally truncated GFP constructs were localized to the mitochondria similar to location of the full length MRPS29 protein in to the mitochondria (Fig. S2). For this reason, it is possible to observe translocation of the full length and/or some of the truncated protein(s) into the mitochondria when uORF-containing MRPS29-GFP construct is expressed in cells (Fig. 4B).

Finally, to determine the location of the 23 residue long peptide possibly expressed from the uORF, uORF-GFP construct was transfected into the HeLa cells (Fig. 4C). According to the Western blot analysis of cells transfected with the uORF-GFP construct, the initiation codon in the uORF recognized by the ribosomes and the GFP tagged peptide is expressed in these cells as observed by an increased molecular weight obtained with the GFP antibody (Fig. S3). However, the uORF-GFP protein was shown a diffused expression similar to that of the GFP protein expression from the pEGFP-N1 control vector (Fig. 4C). All these findings described above suggest that the uORF found in the 5'-UTR of the differentially spliced *MRPS29* mRNA can regulate the expression of the full length MRPS29 protein and impair its import into the mitochondria.

3.4. Role of uORF-MRPS29 in MRPS29-induced apoptosis

To determine the role of uORF-MRPS29 in induction of apoptosis, constructs for MRPS29 and uORF-MRPS29 were cloned into the mammalian expression vector pcDNA3.1 (+) (Fig. 5A). Possible translation initiation sites were indicated with arrows. After the transient transfection of these constructs into HEK293T cells, WST-1 cell viability assays were performed. The WST-1 assay is a modified form of the MTT assay, which is based on the cleavage of tetrazolium salt by mitochondrial dehydrogenases in viable cells. Over-expression of uORF-MRPS29 resulted in cell viability values similar to the control while the over-expression of MRPS29 reduced the cell viability by 30% in HEK293T cells (Fig. 5B). We also evaluated the effect of MRPS29 over-expression on mitochondrial proteins synthesis using [³⁵S]-Met pulse labeling of 13 mitochondrially encoded proteins in the presence of cytoplasmic translation inhibitor, emetine (Fig. S4). Consistent with the previous reports, over-expression of MRPS29 did not alter the protein synthesis in mammalian mitochondria (Fig. S4).

Besides the cell viability assays, Western blot analyses using anti-cleaved Poly(ADP-ribose) polymerase (PARP) antibodies was employed to detect induction of apoptosis in cell lines transiently expressing the MRPS29 constructs. PARP is a nuclear DNA binding protein detected in DNA strand breaks and involved in base excision repair. Once PARP is cleaved by caspases, its DNA repair function is impaired and this phenomenon contributes to the destruction of cells. Therefore, cleaved PARP is used as an indicator of apoptosis (Ame et al., 2004). As shown in Fig. 5C, cleaved PARP detected in cells transiently expressing MRPS29; however, cleavage was not induced in cells transfected with uORF-MRPS29 containing construct.

4. Discussion

In addition to their role in oxidative phosphorylation, mitochondria play a crucial role in several major signaling pathways involved in apoptosis. An elevated level of apoptosis is associated with degenerative diseases while the suppression of this process is involved in carcinogenesis and autoimmune diseases (Danial and Korsmeyer, 2004; Nagata, 1997). For this reason, changes in expression levels and/or post-translational modifications of pro- and anti-apoptotic proteins and factors are tightly regulated in cells. One of the pro-apoptotic mitochondrial ribosomal protein, MRPS29, has been known to induce apoptosis in a variety of cell lines (Berger et al., 2000; Kissil et al., 1999; Miyazaki et al., 2004; Takeda et al., 2007). Besides its role in the induction of apoptosis, MRPS29 was also reported to be involved in resistance to apoptosis in invasive glioblastoma and AT cells (Henning, 1993; Mariani et al., 2001). Here, we report the presence of an alternative form of *MRPS29* mRNA that has an uORF in the 5'-UTR (*uORF-MRPS29*) in a variety of human cell lines, and this uORF might regulate the expression of MRPS29 during progression of apoptosis in human cells. Interestingly, the relative level of *uORF-MRPS29* mRNA was found to be 14–20% of the total *MRPS29* mRNA as determined by quantitative real time RT-PCR analyses in the human cell lines used in this

study. Presence of both *MRPS29* and *uORF-MRPS29* mRNAs might be a significant indication for the regulation of *MRPS29* expression in response to a wide variety of apoptotic stimuli in different cell lines. When the initiation codon in this uORF is recognized by ribosomes for translation initiation, the first AUG of the *MRPS29* coding sequence will overlap with the stop codons found at the 3' end of the uORF. Recognition of the first AUG of the *MRPS29* coding sequence will be impaired in *uORF-MRPS29*; therefore, a decrease in expression of the *MRPS29* will occur. Data presented in this study suggests that the *MRPS29* expression is impaired due to the uORF found in the 5'-UTR of *MRPS29* mRNA as shown by luciferase assays and Western blot analyses. As previously observed, reduction in endogenous *MRPS29* expression by siRNA treatment prevented HeLa and CHO cells to undergo STS-induced mitochondrial fragmentation and apoptosis (Mukamel and Kimchi, 2004). Therefore, reduction of *MRPS29* expression by this uORF may cause resistance to *MRPS29*-induced apoptosis. In fact, this could be one of the possible explanations for resistance to streptonigrin- and ionization radiation-induced apoptosis in AT cells when the uORF containing *MRPS29* mRNA (GenBank Acc. # U18321) is over-expressed in these cell lines (Henning, 1993). In the presence of these findings, we propose that due to the decrease in the expression of *MRPS29* by this uORF, cells transfected with uORF containing construct would not undergo *MRPS29*-induced apoptosis since *MRPS29* is not exceedingly above the endogenous *MRPS29* level.

In addition to *MRPS29*, there is also growing evidence that suggests the involvement of mitochondrial translation activity in apoptosis and cancer (Chen et al., 2009; Chintharlapalli et al., 2005; Kissil et al., 1999; Koc et al., 2001c; Levshenkova et al., 2004; Mariani et al., 2001). Although defects in expression of mitochondrial ribosomal proteins or mutations in these proteins affect the mitochondrial translation severely (Chen et al., 2009, Au and Scheffler, 1997; Toivonen et al., 2003), we believe some of these proteins involved in other functions in the mitochondria, such as apoptosis, may not be related to their roles in protein synthesis. In our cryo-EM studies, we have shown that the most of the mitochondrial ribosomal proteins without bacterial homologs, such as *MRPS29*, localized to the outer surface of the ribosome (Sharma et al. 2003). Interestingly, our results have shown that the overall mitochondrial translation was not significantly affected by the over-expression of *MRPS29* in human cell lines (Fig. S4). Similar findings for the effect of *MRPS29* over-expression and knock down on mitochondrial protein synthesis were also reported by Kimchi et al. (Mukamel and Kimchi, 2004). These observations maybe related to *MRPS29*'s position in the ribosome as it is found to be located towards the lower portion of the small subunit away from the substrate binding sites in the immuno-EM studies (O'Brien et al., 2005). Interaction of the *MRPS29* with the human nitric oxide associated protein 1 (hNOA1), and therefore, Complex I in the inner membrane of mitochondria was reported recently (Tang et al., 2009). Therefore, it may not be functionally critical for peptide synthesis activity but may be for interaction of the mitochondrial ribosome with the inner membrane of mitochondria. All these observations suggest that the pro-apoptotic function of *MRPS29* might not be related to its direct function in translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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MRPS29	1	CCTTTTTCAGTCTCAGGACGGGCGCTTTGGAGCCGGCCCCAGGCAGCGTGTGTCCGGTCG
uORF-MRPS29	1	-----GCAGTCTCAGGACGGGCGCTTTGGAGCCGGCCCCAGGCAGCGTGTGTCCGGTCG
MRPS29	61	CCTAGGCTGGAGAACTAGTCCTCGACTCAGC-----
uORF-MRPS29	54	CCTAGTCTGGAGAACTAGTCCTCGACTCACGGTGAGGGAATGGACCGACACGGGTATTGT
		M D R H G Y C
MRPS29	92	-----TGCAAGGATGATGCTGAAAG
uORF-MRPS29	114	ACCGCTGAGGGGAAGGAGCGGGACTCCGGACCTCCAGGAGTGCAAGGATGATGCTGAAAG
		T A E G K E R D S G P P G V Q G * C *
MRPS29	112	GAATAACAAGGCTTATCTCTAGGATCCATAAGTTGGACCCTGGGCGTTTTTTTACACATGG
uORF-MRPS29	174	GAATAACAAGGCTTATCTCTAGGATCCATAAGTTGGACCCTGGGCGTTTTTTTACACATGG
		G I T R L I S R I H K L D P G R F L H M
MRPS29	172	GGACCCAGGCTCGCCAAAGCATTGCTGCTCACCTAGATAACCAGGTTCCAGTTGAGAGTC
uORF-MRPS29	234	GGACCCAGGCTCGCCAAAGCATTGCTGCTCACCTAGATAACCAGGTTCCAGTTGAGAGTC
		G T Q A R Q S I A A H L D N Q V P V E S

1A

1C

1D

1E

1E

1D

1E

1E

1C

1D

1E

1E

1D

1E

1E

Figure 1.

Presence of uORF in human *MRPS29* transcripts. **(A)** Alignment of 5'-end sequences of alternatively spliced cDNAs obtained from human EST database searching. cDNAs for *MRPS29* and uORF containing *MRPS29* were shown as *MRPS29* and uORF-*MRPS29*, respectively. Protein products possibly encoded from *MRPS29* and uORF-*MRPS29* sequences were indicated above and below cDNA sequences, respectively. (*) represented the stop codons found at the 3' end of the uORF. **(B)** RT-PCR analyses of *MRPS29* and uORF-*MRPS29* transcripts in human cells. cDNAs for HeLa (H), Jurkat (J) and CV-1 (C) were synthesized using 200 ng of total RNA extracted from each cell line. PCR products obtained for *MRPS29*, uORF-*MRPS29* and GAPDH were resolved on a 1.5% agarose gel with a size marker (S). PCR with water (W) was also resolved on the gel for negative control. The gel was stained with ethidium bromide to visualize. **(C)** Quantitation of *MRPS29* variants in human cell lines using real-time PCR. The mRNA levels for *MRPS29* and uORF-*MRPS29* were measured as described in Materials and Methods, and the value obtained for each gene was normalized by one of the housekeeping genes, *cyclophilin A*. **(D)** To determine the ratio of *MRPS29* to uORF-*MRPS29*, amplification efficiency was verified using the known copy number of *MRPS29* and

uORF-MRPS29 plasmids in PCR reactions. After confirming the same amplification efficiency, *uORF-MRPS29* and *MRPS29* levels in different cell line were normalized using *uORF-MRPS29* levels in HeLa cells. The ratio between the *MRPS29* and *uORF-MRPS29* mRNAs in each cell line was calculated as the relative fold and compared to the *uORF-MRPS29* mRNA level in HeLa cells. Relative ratio of *MRPS29* and *uORF-MRPS29* transcripts was shown with black and open bars, respectively. (E) Western blot analysis of endogenous *MRPS29* levels in human cell lines. Approximately 20 μ g of cell lysate from each cell lines were loaded onto a 12% SDS-PAGE. Western blot analysis was performed with anti-*MRPS29*, anti-HSP60 and anti- β -Actin antibodies.

2A

2B

Figure 2.

Recognition of the initiation codon in the 5'-UTR of the *MRPS29* transcript. (A) Schematic diagram of the constructs used in luciferase reporter assays. The expected initiation codon recognition site and direction of translation were shown with arrows. The 5'-UTR region which contained uORF-MRPS29 and initiation codon for luciferase was shown as striped box and closed box, respectively. (B) CV-1 cells were transfected with pRL only (NO), pRL + pGL3-control (pGL3), pRL + pGL3-uORF (pGL3-uORF), and pRL + pGL3-mutant-uORF (pGL3-muORF) using Lipofectamine 2000. The pRL-TK and pRL-SV40 plasmids which produced *Renilla* luciferase were used for normalization to reduce differences in transfection efficiency and variations during experiments. Activity of luciferase was measured by a dual-luciferase assay system. The results represented the mean $\pm$ SD from three independent experiments and were expressed as average luciferase value after normalization. Values of $\star P < 0.05$ were considered statistically significant. ANOVA's *t* test was used to compare the significance of values.

3A

3B

Figure 3.

Down regulation of MRPS29 expression in the presence of the uORF. (A) Design of the MRPS29 and uORF-MRPS29 constructs to detect expression of N-terminally FLAG tagged uORF peptide and C-terminally His-tagged MRPS29 cloned into the pcDNA3.1(+) vector. (B) Western blotting analysis of regulation of MRPS29 expression by the uORF. The HEK293T cells were transfected with 4 μ g of plasmid DNA in a 6-well plate for 48 h. Protein samples (20 μ g of each) obtained from lysing of transfected cells were loaded on a 12% SDS-PAGE. Western blotting probing with anti-HSP60 antibody were employed to ensure equal loading amounts in the gel as a loading control. Changes in MRPS29 expression were detected by anti-MRPS29 and anti-His-tag antibodies in HEK293T cells transfected with pcDNA 3.1 (+) (Control); His-tagged MRPS29 (MRPS29) and C-terminally His-tagged uORF-MRPS29 (uORF-MRPS29) constructs. Recombinant His-tagged MRPS29 protein purified from *Escherichia coli* (Rec-MRPS29) was used as a control for detection with anti-His-tag antibody.

A MRPS29-GFP **B uORF-MRPS29-GFP**

Figure 4.

Role of uORF in translocation of MRPS29 into the mitochondria. Localization of GFP fusion products expressed from MRPS29-GFP (**A**), uORF-MRPS29-GFP (**B**), and uORF-GFP (**C**) constructs were visualized by confocal microscopy. Some of the cells transfected with the uORF-MRPS29-GFP constructs were translocated into mitochondria (indicated by arrow heads), while the others ubiquitously expressed the GFP fusion protein in the cytosol (**B**). Similarly, the 23-residue long peptide expressed from the uORF-GFP construct did not specifically localize the GFP fusion protein into the mitochondria (**C**). HeLa cells were transfected with GFP fusion constructs (green) and stained with MitoTracker Red CMXRos (red) and DRAQ5 (blue) to visualize localization of MRPS29-GFP products, mitochondria,

and nuclei, respectively. Morphology of cells were imaged in the bright field and merged with the GFP images (GFP+Bright). Images were examined using Olympus FluoView 300 confocal laser scanning microscope and merged digitally (Merged).

5A

5B

5C

5C

Figure 5.

Role of uORF in induction of apoptosis. **(A)** Schematic diagram of the MRPS29 constructs cloned into pcDNA3.1 (+) to perform cell viability assays and detection of PARP cleavage by Western blotting as a measurement of apoptotic cell death. Possible translation initiation sites were indicated with arrows. **(B)** Cell viability assays(WST-1) were performed after transfection of 0.2 μ g of each construct in 96-well plate with HEK293T cells. The results represented the mean $\pm$ SD from four independent experiments and were expressed as the percent of control (N/A). Values of $\star P < 0.05$ were considered statistically significant between control and experiment group. ANOVA's *t* test was used to compare the significance of values. **(C)** Changes in MRPS29 expression and cleaved PARP levels were detected by anti-MRPS29 and anti-cleaved PARP antibodies in HEK293T cells transfected with no plasmid (293T); pcDNA 3.1 (+) (pcDNA); pcDNA-uORF-MRPS29 (uORF-MRPS29); pcDNA-MRPS29 (MRPS29); constructs. Western blot analyses were also performed with anti- β -actin and anti-HSP60 antibodies to ensure equal loading amounts were in the gel. About 20 μ g of cell lysates were loaded on a 10% SDS-PAGE.