Journal of Cellular Biochemistry 100:981–990 (2007)

Mitochondrial Ribosomal Protein S36 Delays Cell Cycle Progression in Association with p53 Modification and p21$^{WAF1/CIP1}$ Expression

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Abstract Ribosomal biogenesis is correlated with cell cycle, cell proliferation, cell growth and tumorigenesis. Some oncogenes and tumor suppressors are involved in regulating the formation of mature ribosome and affecting the ribosomal biogenesis. In previous studies, the mitochondrial ribosomal protein L41 was reported to be involved in cell proliferation regulating through p21$^{WAF1/CIP1}$ and p53 pathway. In this report, we have identified a mitochondrial ribosomal protein S36 (mMRPS36), which is localized in the mitochondria, and demonstrated that overexpression of mMRPS36 in cells retards the cell proliferation and delays cell cycle progression. In addition, the mMRPS36 overexpression induces p21$^{WAF1/CIP1}$ expression, and regulates the expression and phosphorylation of p53. Our result also indicate that overexpression of mMRPS36 affects the mitochondrial function. These results suggest that mMRPS36 plays an important role in mitochondrial ribosomal biogenesis, which may cause nucleolar stress, thereby leading to cell cycle delay. J. Cell. Biochem. 100: 981–990, 2007. © 2006 Wiley-Liss, Inc.

Key words: mitochondrial ribosomal protein S36; p21$^{WAF1/CIP1}$ expression; p53 modification; cell cycle delay

Ribosomal biogenesis is correlated with cell cycle, cell proliferation, cell growth, tumorigenesis [Ruggero and Pandolfi, 2003], and consumes up to 80% of the energy of a proliferating cell. As the major component of cell growth, ribosomal biogenesis could conceivably be the target of a checkpoint pathway for monitoring cell growth and coupling a growth condition change or insult to cell cycle [Thomas, 2000; Pestov et al., 2001]. Ribosomal proteins play an important role in ribosomal biogenesis in response to some stresses. Several ribosomal proteins have been determined to activate p53 by inhibiting the MDM2/HDM2-mediated feedback regulation of p53, leading to cell cycle arrest via p53 stabilization [Zhang et al., 2003; Bhat et al., 2004; Dai and Lu, 2004; Dai et al., 2004]. Moreover, the expression of cyclin-dependent kinase (CDK) inhibitor p21$^{WAF1/CIP1}$ has been shown to be involved in a p53-dependent cross-talk between ribosome biogenesis and cell cycle progression [Pestov et al., 2001].

Mitochondria have been shown to be important in a death regulator in response to DNA damage, growth factor withdrawal, hypoxia, and anticancer drug therapy [van Gurp et al., 2003; Orrenius, 2004]. The proper expression of the mitochondrial encoded protein genes depends on the nuclear encoded components of the mitochondrial translation system. Mitochondrial ribosomal proteins (MRPs) are required for the translation of all 13 mitochondria gene [Poyton and McEwen, 1996; Scarpulla, 1997]. The MRPs, death-associated protein 3 (DAP3), and the programmed cell death protein 9 (PDCD9) were shown to be involved in

Grant sponsor: National Science Council, Taiwan; Grant numbers: NSC93-2320-B-006-080, NSC92-2320-B-006-095.

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Received 23 May 2006; Accepted 26 June 2006

DOI 10.1002/jcb.21079

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apoptosis [Cavdar et al., 2001b]. The previous studies reported that the mitochondrial ribosomal protein MRPL41, which is encoded by nuclear genes, contributed to p53 stability and induced apoptosis. Furthermore, MRPL41 was also been shown to cause cell cycle arrest at G1 phase. Therefore, the MRPs play an important role in cell growth regulation [Chintharlapalli et al., 2005; Kim et al., 2005; Yoo et al., 2005].

Here, we performed cDNA representational difference analysis (RDA) to identify genes that were differentially expressed between mouse NIH3T3 fibroblasts and its prothymosin α (ProT) overexpression derivative, pp23. ProT is an acidic, ubiquitous protein that contains 113 amino acid residues with thymosin αl sequence at its N-terminus [Segade and Gomez-Marquez, 1999]. ProT is a nuclear protein known to play an essential role in the proliferation of mammalian cells [Wu et al., 1997]. Recently, ProT was reported to inhibit apoptosome formation and regulated mitochondrial-mediated apoptosis. In apoptotic cells, the caspases were activated and ProT was cleaved by caspases [Enkemann et al., 2000; Evstafieva et al., 2000, 2003; Jiang et al., 2003]. Although ProT is an oncoprotein required for cell proliferation, the biological functions and the identities of downstream effectors were not clear. We therefore sought to identify genes that act downstream of ProT in a biologically relevant system. In our present study, we describe the identification and characterization of a putative ProT target gene in pp23 cells, termed the mouse mitochondrial ribosomal protein S36 (mMRPS36), which has 84.15% identity with *Homo sapiens* mitochondrial ribosomal protein S36 (MRPs36) (*NM_033281*). Interestingly, we discovered that overexpression of mMRPS36 could retard the cell proliferation. We attempted to ascertain whether the overexpression of mMRPS36 could delay the cell cycle progression. We also attempted to evaluate the possible involvement of $p21^{WAF1/CIP1}$, activated by p53, with regard to the cell cycle delay caused by mMRPS36 overexpression.

MATERIALS AND METHODS

Cell and Cell Culture

Cells were cultured in the complete medium containing Dulbecco's modified Eagles medium (DMEM) supplemented with 10% calf serum, 2 mM L-glutamine, 20 mM HEPES, and 50 μg/ml gentamicin at 37°C in 5% CO_2. pp23 cells were derived from mouse NIH3T3 cells stably transfected with mouse ProT gene [Wu et al., 1997].

Representational Difference Analysis (RDA)

A complete protocol that details each step in the RDA method was described previously [Pastorian et al., 2000]. pp23 cells were derived from mouse NIH3T3 cells stably transfected with mouse ProT gene [Wu et al., 1997]. Total RNA was isolated from NIH3T3 and pp23 cells using RNA isolation kit (MBI, Ontario, Canada) and the mRNA from total RNA was purified using Oligotex mRNA Midi kit (QIAGEN, Hilden, Germany). The mRNA samples were used for RDA by CLONTECH PCR-Select cDNA Subtraction kit (Clontech, Mountain View, CA). Upon completion of RDA procedure, the differentially expressed products could be obtained and sequenced.

Generation of mMRPS36-overexpression NIH3T3 Cells and Detection of Transgene Expression in the Transfectants

The coding region of mMRPS36 was cloned into the CMV promoter-controlled vectors, pcDNA3.1-cmyc and pEGFP-N1 (Clontech), and also into the rat β-actin promoter-driven plasmid pTCY [Shiau et al., 2002], yielding mMRPS36-Myc, mMRPS36-EGFP, and mMRPS36, respectively. NIH3T3 cells were transfected with mMRPS36 DNA by calcium phosphate method or Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and clonal derivatives were isolated by G418 selection (750 μg/ml) and expanded to independent clones. Likewise, a vector control was isolated and used for parallel studies.

Immunohistochemistry

Cells were cultured on microscope slides, fixed in 10% formaldehyde and permeabilized with cold acetone. Samples were incubated with anti-mMRPS36 antibody at 4°C overnight, washed with phosphate-buffered saline (PBS), and then incubated with the appropriate secondary antibody conjugated with alkaline phosphates (DAKO) at 4°C for 4 h. Signals were detected by AEC substrate kit (ZYMED, S. San Francisco, CA).

Confocal Microscopy

The pEGFP-N1 or mMRPS36-pEGFP plasmid DNA was transfected into 293 cells. Forty-eight hours later, cells were fixed in 3.7% paraformaldehyde, washed with PBS, and stained for 30 min with 100 nM MitoTracker Red CMXRos (Molecular Probes, Willow Creek Road Eugene, OR). The mitochondria were visualized using a confocal microscope equipped with 506-nm and 600-nm laser beams.

Proliferation Analysis

Cells either from the stable clones or transiently transfected cells were seeded in 96-well plates. After 72 h, cells were labeled with [H^3]-thymidine (1 μCi/well) (Amersham) and incubated for 16 h. After incubation, cells were harvested onto fiberglass filters (Skatron) using Filter Mate cell Harvester (Packard), and counted using a MATRIX™ 9600 direct β counter.

Cell Viability Assay

Cells were seeded in 24-well plates, and methyl thiazol tetrazolium assay (MTT assay; Sigma) was performed for cell growth analysis. Cells were incubated with 0.25 mg/ml MTT for 4 h at 37°C and the reaction was terminated by the addition of 100% iso-propanol. The amount of MTT fromazon product was determined by a microplate reader and the absorbance was measured at 570 nm (SpectraMax 250, Molecular Devices, Sunnyvale, CA).

Cell Cycle Analysis

Cells were cultured in 6-well plates, and serum starved in DMEM supplemented with 0.1% calf serum. After serum starvation for 48 h, 10% serum was added to cells, and cells were harvested at the indicated times. Cell were fixed in cold 70% ethanol for 30 min. Mixtures were centrifuged at 2,000 rpm for 10 min at 4°C, washed two times with PBS, and added with RNaseA (100 μg/ml) for 20 min at room temperature. Propidium iodide (400 μg/ml) was subsequently added and incubated for at least 20 min at room temperature and were analyzed on a FACScaliber flow cytometry (Becton Dickinson, Franklin Lakes, NJ). The experiment was repeated three times.

Luciferase Activity Assay

Cells were transfected with 1 μg of the p53-luciferase plasmid (Stratagene, La Jolla, CA) and 0.2 μg of pTCY-Laz, a β-galactosidase reporter vector, by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48-h incubation, cells were harvested and lysed in Tropix lysis solution (Tropix, Bedford, MA), and the luciferase activities of cell lysates were measured by Dual-luciferase Reporter Assay System (Tropix). The luciferase activity was expressed as relative light units (RLU)/μg protein. Protein concentration of the cell lysates was measured using the BCA assay (Pierce, Rockford, IL).

Preparation of the Cytosolic, Mitochondria and Total Proteins

The pcDNA3.1-cmyc or mMRPS36-Myc plasmid was transfected into 293 cells. Cells were harvested in lysis buffer A without SDS (50 mM Tris-HCl, 4 mM EDTA, 2 mM EGTA). Lysates were sheared by passing them five times through a 27-gauge needle and centrifuged at 3,000 rpm for 10 min at 4°C. Lysates were then centrifuged at 14,000 rpm for 40 min at 4°C. The supernatant, the cytosolic extract, and the pellet, the mitochondria extract, were suspended in cell lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1× protease inhibitors cocktails (Pierce)]. For the total cell lysates, cells were harvested by trypsinization, washed with PBS (pH 7.4), and centrifuged at 3,000 rpm for 10 min at 4°C. Cell were then lysed with 50 μl of an ice-cold cell lysis buffer and subjected to centrifugation at 12,000 rpm for 20 min at 4°C. Protein concentrations were determined using the BCA protein Assay (Pierce).

Western Blot Analysis

Cell lysates (10–30 μg) were resolved on SDS–PAGE gel and transferred to nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech). Non-specific binding was blocked using 5% non-fat milk or 5% BSA/TBS for 60 min, washed four times, for 8 min each, with Tris-buffered saline (TBS; pH 7.4), 0.1% Tween-20, and incubated with the primary antibody overnight at 4°C. After washing, the membrane was incubated with a secondary antibody conjugated with alkaline phosphates for 1 h at room temperature. Membrane was washed four times with TBS (pH 7.4) and 0.1% Tween-20 for 8 min. Bands were visualized using a peroxidase-linked enhanced chemiluminescence detection system (ECL, Amersham

Pharmacia Biotech). The monoclonal anti-p21$^{WAF1/CIP1}$ (F-5, Santa Cruz Biotechnology), monoclonal anti-Myc (9E10, Santa Cruz Biotechnology), monoclonal anti-GFP (B-2, Santa Cruz Biotechnology), monoclonal anti-p53 (Ab3, Oncogene), anti-phospho-p53 (serine 15) (Cell Signaling-NEB, 9284S) and monoclonal anti-β-actin (AC-74, Sigma) antibodies were used in the study.

Measurement of Mitochondrial Membrane Potential

Mitochondrial function was indirectly assessed by variation in mitochondrial transmembrane potential measured by rhodamine 123 (Sigma) [Mancini et al., 1997]. Cells were incubated with 0.5 μg/ml of rhodamine 123 for 30 min at 37°C incubator, and then trypsinized, washed by PBS and immediately submitted for flow-cytometric analysis (FACScaliber; Becton Dickinson).

Measurement of Reactive Oxygen Species (ROS)

Intracellular oxidative products were detected by H_2DCF-DA as an intracellular fluorescence probe. Briefly, the vector or mMRPS36 overexpression stable clones, and the chloramphenicol-treated NIH3T3 cells, were treated with H_2DCF-DA (50 mM) for 30 min at 37°C. Cells were collected and analyzed immediately by FACSCalibur (Becton Dickinson).

RESULTS

Identification of mMRPS36

Mouse NIH3T3 and pp23 cells were used for RDA, and several distinct PCR fragments unique for pp23 cells were obtained (data not shown). Seven DNA fragments were expressed with high frequency. One of the fragment sequences revealed 100% identity with the *Mus musculus* RIKEN cDNA 1110018B13 gene [Kawai et al., 2001] (GeneBank accession no, *AK003765*), designated MRPS36, by the BLAST database search (NCBI). mMRPS36 cDNA is 309 bp in length, and encodes a 102-amino-acid peptide (Fig. 1A). The amino acid sequence alignment revealed that there were 86.13% similarity and 84.15% identity between mouse MRPS36 (*NP_079645*) and *Homo sapiens*

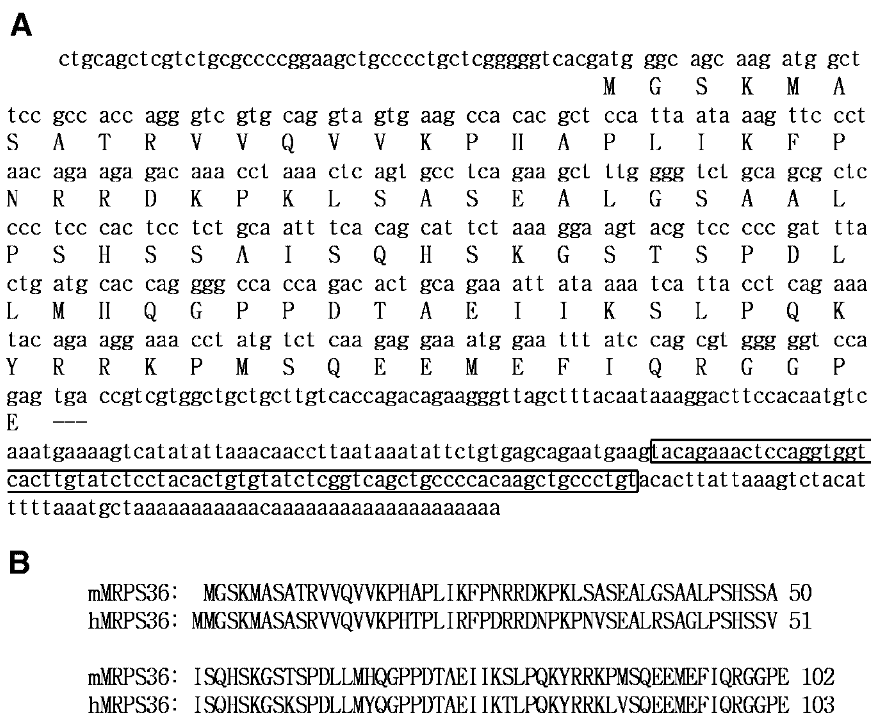

Fig. 1. cDNA and protein sequences of mMRPS36. **A**: Sequence of the mMRPS36 cDNA (*BC028499*). The open box represents the sequence from the RDA result. **B**: Amino acid sequence alignment of mouse MRPS36 protein (*NP_079645*) and *Homo sapiens* mitochondrial ribosomal protein S36 (MRPs36) (*NM_033281*). The alignment was performed using the SeqWeb program. m, mouse; h, human.

mitochondrial ribosomal protein S36 (MRPs36) (*NM_033281*) (Fig. 1B) [Cavdar et al., 2001a].

mMRPS36 was Located in the Mitochondria

In order to determine the localization of mMRPS36, the green fluorescent protein-tagged mMRPS36 gene was introduced to 293 cells, and its distribution in cells was observed. The mMRPS36 expression was detected in the cytoplasm (Fig. 2A). The mitochondrion-specific dye MitoTracker was also used. Signals of the red MitoTracker and the green mMRPS36-EGFP were overlapped. This demonstrates that the mMRPS36 protein is localized in the mitochondria (Fig. 2A). In order to further confirm the localization of mMRPS36 protein, the mitochondrial fractional experiment was performed with 293 cells transfected with the Myc-tagged mMRPS36 plasmid (mMRPS36-Myc). Control cells were transfected with the Myc vector. As expected, mMRPS36 protein was detected in the mitochondrial fraction of 293 cells which were transfected with mMRPS36-Myc, while cytochrome c as the mitochondria marker, and β-actin as the cytoplasm marker (Fig. 2B). These results demonstrated that mMRPS36 was expressed in the mitochondria.

mMRPS36 Overexpression Retarded Cell Growth and Proliferation, and Delayed the Cell Cycle Progression

To understand the functions of mMRPS36 in cells, the expression vector, pTCY-mMRPS36, under the control of the rat β-actin promoter was constructed. The plasmid was transfected into NIH3T3 cells and stable expression clones were selected by G418. The overexpression of mMRPS36 in stable transfectants was confirmed by RT-PCR (Fig. 3A) and immunohistochemical staining (Fig. 3B). In order to determine whether the mMRPS36 plays a role in cell growth, MTT assay (Fig. 3C) and [H^3]-thymidine incorporation (Fig. 3D) were performed in the vector- and mMRPS36-modified cells. Surprisingly, the mMRPS36-modified clones showed significantly lower growth rate and proliferation than the vector control. A consistent result was observed in cells transiently transfected with the mMRPS36, and showed a dose-dependent retardation of proliferation with the mMRPS36 gene expression (Fig. 3E). To determine whether mMRPS36 regulates the cell cycle progression, effects of mMRPS36 on the cell cycle were investigated. After synchronization by serum starvation, mMRPS36

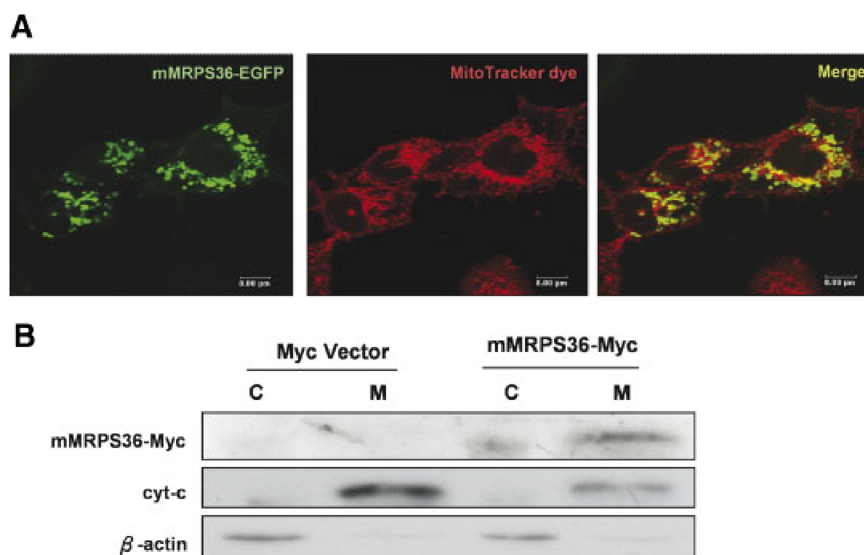

Fig. 2. The localization of mMRPS36 was in the mitochondria. **A**: The EGFP fusion protein of mMRPS36 was transiently expressed in 293 cells, counterstained with the MitoTracker probe, and imaged by a confocal microscopy. Mitochondria were stained with MitoTracker Red CMXRos dye. **B**: Western blot analysis of mitochondrial and cytosolic extracts (10 μg/lane) of 293 cells transiently transfected with the Myc-tagged mMRPS36 or Myc vector control (pcDNA3.1/myc-His). Fractions were separated, transferred onto membranes, and incubated with anti-Myc antibody. Membranes were then reblotted with anti-cytochrome c (cyt-c, the mitochondria marker; BD PharMingen), or the cytoplasm marker, anti-β-actin antibody. C, cytosolic fraction; M, mitochondrial fraction. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

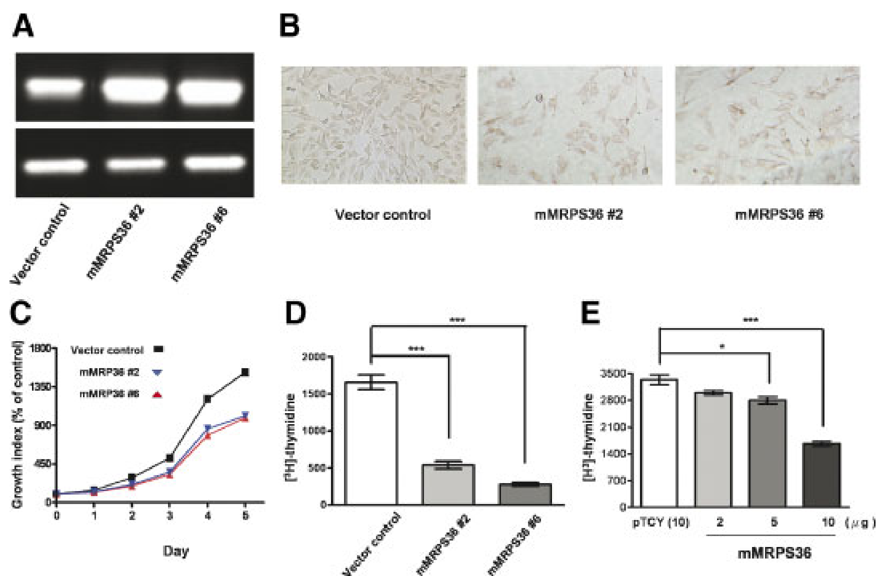

Fig. 3. Overexpression of mMRPS36 retarded cell growth and cell proliferation. Overexpression of the mMRPS36 was confirmed by RT-PCR (**A**) and immunohistochemical staining (**B**, original magnification ×200). Cells were cultured on microscope slides and incubated with the rabbit anti-mMRPS36 serum and then the second antibody (anti-rabbit) conjugated with alkaline phosphatase. Signals were detected by AEC substrate kit. **C**: The vector or mMRPS36 overexpression cells were seeded on 24-well plates at a density of 1×10^4 cells per well in culture medium, and cell growth was detected by MTT assay. **D**: Cells (500 per well) were cultured in 96-well plates. After 3 days, cells were labeled with [H^3]-thymidine. Data were expressed as mean ± standard deviation of the mean (SD) of ten determinations. **E**: The pTCY (10 μg), or the mMRPS36 (2, 5, or 10 μg, respectively) plasmid was transiently transfected into NIH3T3 cells. Cell proliferation was determined by [H^3]-thymidine incorporation after transfection. Data were expressed as mean ± SD of ten determinations. (*$P < 0.05$; ***$P < 0.001$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

overexpression cells showed a cell cycle delay for 6 h (Fig. 4). This indicated that mMRPS36 retarded NIH3T3 cell proliferation by affecting the cell cycle at the G1 phase.

mMRPS36 Induced the $p21^{WAF1/CIP1}$ Expression and p53 Post-Translational Modification

As the mMRPS36 gene-modified cells showed that a cell cycle delay for 6 h, in order to ascertain the underlying mechanism, cell cycle regulators induced by mMRPS36 overexpression were examined. It was well known that the tumor suppressor p53 is a key regulator of both the cell cycle and cell proliferation. Therefore, the p53 transcriptional activity and protein expression in mMRPS36 overexpression cells were analyzed. The results showed that p53 transcriptional activity (Fig. 5A) and p53 expression level (Fig. 5B) were elevated by the mMRPS36 overexpression. The post-translational modifications usually drive p53 transcriptional activation and generally result in p53 stabilization, accumulation and activation induced by cellular stresses, and it has also been reported that phosphorylation at serine 15 of p53 is associated with its transcriptional activity [Jaiswal and Narayan, 2002; Bode and Dong, 2004]. Therefore, the phosphorylation status at serine 15 was analyzed. Higher phosphorylation levels of p53 at serine 15 were observed in the mMRPS36-modified cells as compared with the control (Fig. 5B). To verify the induction of p53 by mMRPS36, HepG2 cells were transiently transfected with mMRPS36-EGFP, and then levels of p53 expression and phosphorylation status were assessed. p53 protein levels and phosphorylation at serine 15 were gradually increased in mMRPS36-EGFP-transfected cells, in which increasing mMRPS36 expressions were detected by the anti-GFP antibody (Fig. 5C). As $p21^{WAF1/CIP1}$ plays an important role in cell cycle arrest and its expression has been shown to be regulated largely at the transcriptional level by p53-dependent mechanism [Gartel and Tyner, 1999; Jaiswal and Narayan, 2002], we also examined the expression of $p21^{WAF1/CIP1}$. The $p21^{WAF1/CIP1}$ expression was found to be increased in the mMRPS36 overexpression cells by Western blot analysis (Fig. 5B), and was shown to be regulated by the p53 expression (Fig. 5C). Overall, these results demonstrated that mMRPS36 induced

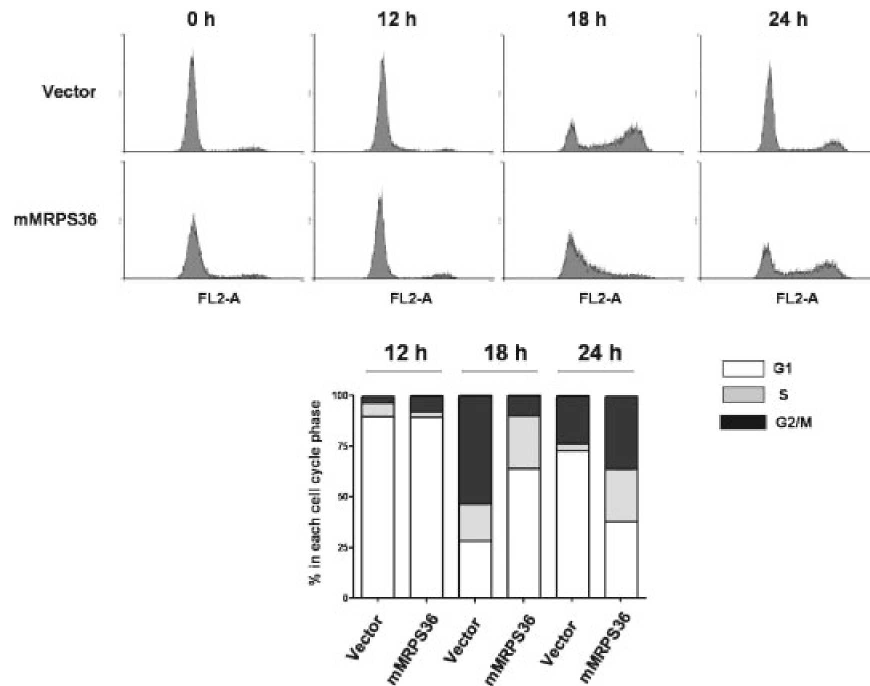

Fig. 4. mMRPS36 overexpression delayed cell cycle progression. mMRPS36 overexpression cells of clone #6 were synchronized by serum deprivation (with 0.1% calf serum). Cells arrested at G1 were stimulated with 10% fetal calf serum to re-enter the cell cycle. Cells were stained with propidium iodide and processed by FACS analysis. The **upper panel** shows the cell cycle profile of cells at 0, 12, 18, and 24 h after serum stimulation. The proportion of cells in each cell-cycle phase is presented in the **lower panel**. Experiment was repeated three times.

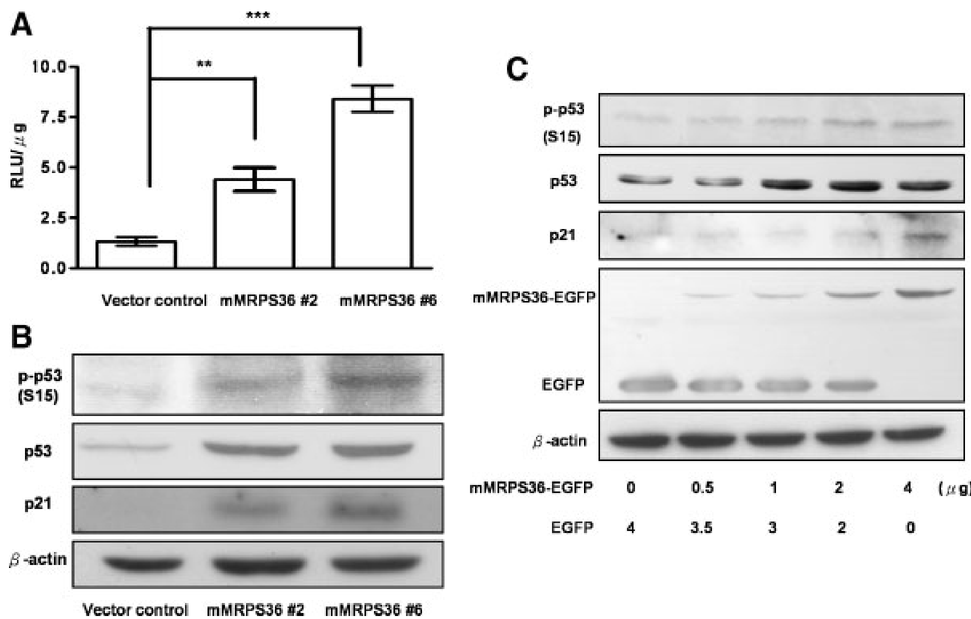

Fig. 5. mMRPS36 induced the p21$^{WAF1/CIP1}$ expression and p53 post-translational modification. **A**: Transcription activity of p53 in mMRPS36 overexpression cells. Cells were transfected with p53-luciferase plasmid and the internal control vector, pTCY-Laz. After 48 h, cells were harvested and lysed, and the luciferase activities were measured (**$P<0.01$; ***$P<0.001$). **B**: Western blot analysis of mMRPS36 overexpression cells. Cells lysates (30 μg/lane) were resolved via SDS–PAGE, and Western blot analysis was performed with the anti-p53, anti-phospho-p53 (S15), anti-p21$^{WAF1/CIP1}$, or anti-β-actin antibody. **C**: Increasing amounts of the mMRPS36-EGFP were transfected into HepG2 cells and Western blot analysis was performed with the anti-p53, anti-phospho-p53 (S15), anti-p21$^{WAF1/CIP1}$, anti-GFP or anti-β-actin antibody.

cell cycle arrest via affecting the expression and post-translational modification of p53, and regulating the expression of $p21^{WAF1/CIP1}$.

mMRPS36 Expression Affected the Mitochondria Function and ROS Generation

It has been shown that MRPs are required for the translation of all 13 mitochondria genes. Consequently, we examined whether mMRPS36 affects the mitochondria function by analyzing the mitochondrial membrane potential. In our study, the mitochondrial membrane potential indicated by the uptake of rhodamine 123 was altered in mMRPS36 overexpression cells (Fig. 6A,B). It was reported that the mitochondrial membrane potential disruption could induce transient cell cycle arrest through the p53 and $p21^{WAF1/CIP1}$ expression and increase mitochondrial ROS generation [Pluquet and Hainaut, 2001; Barnouin et al., 2002; Yoon et al., 2003; Boonstra and Post, 2004], hence, the ROS generation in mMRPS36 overexpression cells was also analyzed as described previously [Narayanan et al., 2004], while the ROS production in chloramphenicol-treated NIH3T3 cells as the positive control (Fig. 6C). Chloramphenicol is an antibiotic and toxicity for eukaryotic cells connecting with the inhibition of mitochondrial protein synthesis, and thereby causes ROS generation [Ramachandran et al., 2002]. In our assay, the ROS production was increased (Fig. 6C). This indicated that overexpression of mMRPS36 induced the production of ROS.

DISCUSSION

We applied RDA to cDNAs generated from NIH3T3 cells and its derivative, pp23. Several of the differential fragments identified in our screens. We chose one of these differential fragments, mMRPS36, for our further investigations. Our results showed the mMRPS36 distributed in the cytoplasm and was localized in the mitochondria. The overexpression of mMRPS36 retarded the cell proliferation and delayed cell cycle progression through altering the expression and modification status of p53, and also the expression of $p21^{WAF1/CIP1}$.

Mitochondria are intracellular organelles responsible for ATP synthesis in mammalian cells. Most of the proteins that reside in mitochondrion are nuclear gene products and play important roles in transcription, translation, and replication of mitochondria [Poyton and McEwen, 1996; Lee and Wei, 2000]. MRPs are required for the translation of all 13 mitochondria genes. Proteins which are essential for

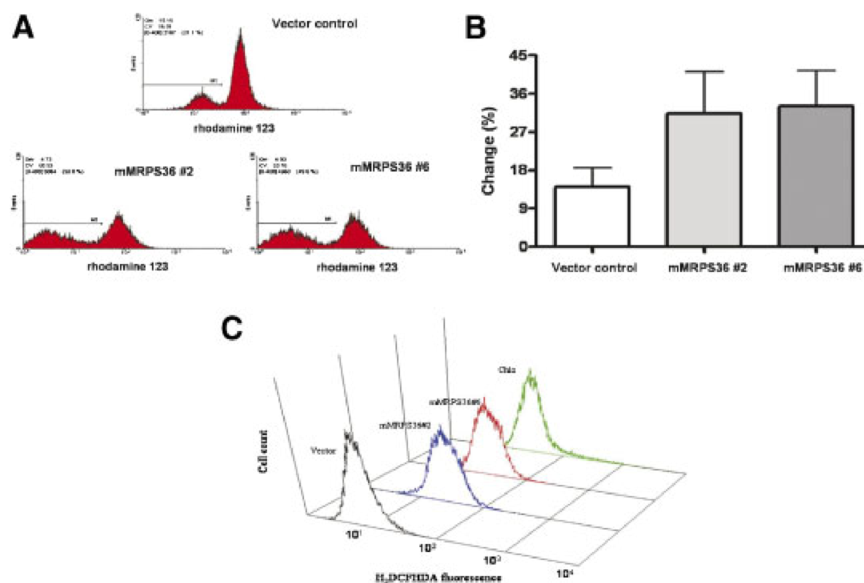

Fig. 6. mMRPS36 affected mitochondrial membrane potential and ROS production. **A**: Histograms generated by FACS analysis of the vector- or mMRPS36-modified cells incubated with rhodamine 123. The X axis represents the rhodamine 123 fluoresce. **B**: Percentage of the mitochondrial membrane potential (MMP) change was shown in the panel. **C**: The vector control cells, mMRPS36 overexpression cells, and chloramphenicol-treated NIH3T3 were incubated with H_2DCFDA at 37°C for 30 min, and intracellular ROS levels were analyzed by flow cytometry. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mitochondrial ribosome formation are candidates for involvement in human genetic disease [O'Brien, 2002; Sylvester et al., 2004]. Ribosomal proteins have shown to precipitate in controlling protein biosynthesis and ribosome biogenesis, and the MRPs are directly involved in this process. A previous study showed the MRPs, DAP3, and PDCD9 may involve in apoptosis [Cavdar et al., 2001b]. However, the function of most MRPs has not been investigated individually. Although the molecular mechanism controlling the link between ribosomal biogenesis and cell cycle regulation remains unclear, several studies suggest that some ribosomal proteins, L5, L11, and L23 may be involved in this regulation. These ribosomal proteins interact with MDM2 and stabilize p53 via inhibiting the MDM2-mediated p53 ubiquitination and degradation process. These results indicate the p53 pathway in a heretofore unknown function as a monitor of ribosome biogenesis and provide the molecular mechanism linking ribosomal biogenesis and cell proliferation in mammalian [Pestov et al., 2001; Zhang et al., 2003; Bhat et al., 2004; Dai and Lu, 2004; Dai et al., 2004]. The mitochondrial ribosomal protein MRPL41 was reported to induce cell cycle arrest at G1 through the accumulation of p53 via inhibiting the MDM2-mediated p53 ubiquitination and degradation process. MRPL41 also induced $p21^{WAF1/CIP1}$ expression to induce cell cycle arrest under growth inhibitory conditions [Kim et al., 2005; Yoo et al., 2005]. In this study, we have identified another mitochondrial ribosomal protein S36, which increased the $p21^{WAF1/CIP1}$ expression through increasing the p53 expression and phosphorylation at serine 15 (Fig. 5B). Modification of phosphorylation sites of p53 stabilizes p53 by inhibiting MDM2-dependent degradation and can increase the cellular level of p53 [Jaiswal and Narayan, 2002].

The main endogenous source of reactive oxygen species (ROS) found in living cells is the mitochondria. ROS was reported to influence cell cycle progression depending upon its production. ROS induced transient growth arrest to permanent growth arrest, to apoptosis or to necrosis, dependent on the level of ROS. The ubiquitination process was inhibited by oxidative stress [Boonstra and Post, 2004]. In addition, sublethal level of ROS caused a transient arrest in NIH3T3 fibroblasts and increased the p53 and $p21^{WAF1/CIP1}$ expression [Barnouin et al., 2002; Boonstra and Post, 2004]. In our result, it was observed that the mMRPS36 overexpression induced ROS production (Fig. 6C). This suggests that mMRPS36 overexpression may influence mitochondria function and ROS production that may be implicated as second messengers between mitochondria and stress-induced p53 activation.

In this study, we identified the mitochondrial ribosomal protein S36 (mMRPS36), which is encoded by a nuclear gene and involved in protein synthesis within the mitochondria. Our study reveals that mMRPS36 employs mechanisms in terms of both the expression and phosphorylation of p53 and the $p21^{WAF1/CIP1}$ expression to delay cell cycle progression and retard cell proliferation. The precise biological function of mMRPS36 in cells remains to be clarified. Further investigations will be required in order to elucidate the mechanisms underlying the cellular regulating functions of mMRPS36.

ACKNOWLEDGMENTS

The authors thank Drs. Ming-Derg Lai, Wen-Tsan Chang, and Yee-Shin Lin for valuable discussions. This work was supported by grants from the National Science Council, Taiwan (NSC93-2320-B-006-080 and NSC92-2320-B-006-095) and by the Foundation of Dr. Chen, Jieh-Chen Scholarship, Tainan, Taiwan.

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