Cytoplasmic ribosomal protein S3 (rpS3) plays a pivotal role in mitochondrial DNA damage surveillance

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ARTICLE INFO

Article history:
Received 4 January 2013
Accepted 17 July 2013
Available online 30 July 2013

Keywords:
HSP70
HSP90
Mitochondria
Ribosomal protein S3
ROS

ABSTRACT

Ribosomal protein S3 (rpS3) is known to play critical roles in ribosome biogenesis and DNA repair. When cellular ROS levels increase, the mitochondrial genes are highly vulnerable to DNA damage. Increased ROS induces rpS3 accumulation in the mitochondria for DNA repair while significantly decreasing the cellular protein synthesis. For the entrance into the mitochondria, the accumulation of rpS3 was regulated by interaction with HSP90, HSP70, and TOM70. Pretreatment with geldanamycin, which binds to the ATP pocket of HSP90, significantly decreased the interaction of rpS3 with HSP90 and stimulated the accumulation of rpS3 in the mitochondria. Furthermore, cellular ROS was decreased and mtDNA damage was rescued when levels of rpS3 were increased in the mitochondria. Therefore, we concluded that when mitochondrial DNA damages accumulate due to increased levels of ROS, rpS3 accumulates in the mitochondria to repair damaged DNA due to the decreased interaction between rpS3 and HSP90 in the cytosol.

1. Introduction

Mitochondria are the major site of energy production in eukaryotic cells. Mitochondria continuously synthesize ATP through oxidative phosphorylation. However, this process generates reactive oxygen species (ROS) that injure the mitochondrial genome, especially during ATP generation. The point mutation disrupts the mitochondrial gene for tRNA-Lys and subsequently dysregulates the synthesis of proteins essential for oxidative phosphorylation. The MT-ATP6 protein forms one subunit of ATP synthase. Vogelstein et al. also reported that increased levels of mtDNA mutations have been detected in colorectal cancers [1].

The mitochondrial matrix contains a complete genetic system. However, only ~1% of mitochondrial proteins critical for ATP synthesis are encoded by the mitochondrial genome and synthesized in the matrix [2]; 99% of mitochondrial proteins are encoded by nuclear genes. Therefore, there is no doubt that all enzymes that repair mtDNA damage must be translocated into the mitochondria from the cytosol. For protein translocation into mitochondria, three basic components are required: a mitochondrial targeting sequence (MTS) on nucleus-encoded mitochondrial precursor proteins, a specific import receptor on the mitochondrial outer membrane, and an Hsp70/Hsp90 chaperone or other soluble factors which guide the protein into the mitochondria [3].

Nucleus-encoded mitochondrial precursor proteins contain targeting signals that are recognized by receptors on the mitochondrial surface. These targeting signals direct the precursors to their functional destinations in the mitochondrial sub-compartment. There are two main groups of targeting signals [4,5]. The first consists of amino-terminal precursor extensions, which are classical MTSs. These pre-sequences are usually removed proteolytically after import into the mitochondria. Alternatively, many precursor proteins are not synthesized with cleavable extensions but contain internal targeting signals that are a part of the mature protein. This second group of signals includes different types of precursor proteins and targeting signals. Currently, at least five main classes of precursor proteins are known with each class having a different import route into the mitochondria.

The common entry gate for most precursors is formed by a translocase of the outer membrane (TOM) complex [3–8]. The TOM complex comprises of a central Tom40 component, three pre-protein receptors (Tom20, Tom22, and Tom70), and several small TOM proteins [3,5,6,8,9]. Major portions of Tom20, Tom22, and Tom70 are exposed on the cytosolic side of the mitochondrial outer membrane and function as import receptors in organelles for distinct classes of pre-proteins in vivo [10]. Purified cytosolic domains of these import receptors are known to specifically bind mitochondrial pre-proteins in vitro [11]. In particular, Tom70 is required for the import of non-cleavable pre-proteins and is specific for carrier proteins destined for the inner mitochondrial membrane [12, 13] and 14-3-3 [14]. Hsp90 and Hsp70 proteins are recognized by a specific tetra-tricopeptide repeat (TPR) motif of import receptors. This recognition facilitates movement of specific pre-proteins to import receptors for Hsp90/Hsp70 ATPase-dependent membrane translocation.
Ribosomal protein S3 (rpS3) is a critical component of the 40S subunit of the ribosome. Recently, it has been established that rpS3 is not only involved in the function related with protein translation, but also had extra-ribosomal activities such as DNA repair [15–19], cell signaling [20–23], apoptosis/survival [24], host-pathogen interactions [25,26], and transcriptional regulation [27–29]. In a transcriptional study, we discovered that rpS3 cleaves the phosphodiester bond between pyrimidine dimers as an important step of excision repair in mammalian and yeast systems [16,19]. We also noted that rpS3 protein possesses a lyase activity specific for damaged DNA containing 8-oxoG, AP sites and thymine glycols. Other recent studies showed that rpS3 binds to 8-oxoG sites containing DNA without showing a glycosylase activity [17,19]. Furthermore, rpS3 also facilitates the repair activity of base excision repair (BER) enzymes such as hOGG1 and APE/Ref-1 [17]. Non-ribosomal rpS3 appears to be protected by interaction with Hsp90 from ubiquitin-dependent proteasomal degradation [30].

In the present study, we found that mitochondrial accumulation of rpS3 was increased by oxidative stress due to mitochondrial DNA damage. Translocation of rpS3 was mediated by the Hsp90-Hsp70-Tom70 receptors. Furthermore, we showed that increased levels of rpS3 protein in the mitochondria reduce cellular ROS concentrations and mtDNA damage. Taken together, our findings suggested that rpS3 plays a critical role in the surveillance of mitochondrial DNA integrity after the cell experiences oxidative stress.

2. Material and methods

2.1. Preparation of whole-cell lysates and mitochondrial fractions

After treatment with curcumin or hydrogen peroxide, HT1080 or HEK293T cells were pelleted by centrifugation and washed once with PBS. The cells were then resuspended in a lysis buffer (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) NP-40, 1 mM sodium glycerophosphate, 10 mM NaF, 1 mM Na3VO4, 0.25% (v/w) deoxycholate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). Whole-cell lysates were prepared by sonication and centrifuged (16,000 × g for 15 min at 4 °C). Mitochondrial and cytosolic fractions were isolated from HT1080 cells or HEK293T cells using a Qproteome™ Mitochondria Isolation kit (Qiagen, Valencia, CA). After the final centrifugation (6,000 × g for 10 min at 4 °C), cytosolic and mitochondrial fractions were recovered from the supernatant and pellet, respectively. The PN (post-nuclear supernatant without nuclei), PN-HM (supernatant without the heavy membrane fraction or nucleus), and mitochondria were isolated with sucrose-density ultracentrifugation as previously described [31]. To separate to mitochondrial inner membrane and matrix fraction, isolated mitochondria were incubated in 15 mM KCl buffer on ice and then mitoplasts were separated with inner membrane and matrix particle by sonication and centrifugation.

2.2. Dot-blot analysis for 8-oxoG detection

After isolation of mitochondria from HEK293T cells with mtDNA damage induced by ROS-inducing agents (H2O2 or curcumin), mtDNAs were purified by DNA purification Kit (Promega) according to the manufacturer’s protocol. The 1 μg of purified DNA was loaded twice onto a nontoccellulose membrane and dried for 15 min. To cross-link DNA, membrane was incubated at 65 °C for 25 min and immunoblot analysis was performed with anti-8-oxoG antibody. As a loading control, mitochondrial 16S rRNA gene probe (Supplemental Table S1) was used for southern blot analysis.

2.3. Sub-cloning for rpS3 over-expression and siRNA transfection

To express rpS3 protein in the mitochondria, the full-length rpS3 gene was inserted into a pCMV/myc/mito plasmid (Invitrogen). To identify the mitochondrial target signal (MTS) in rpS3 protein, full-length and deletion mutant rpS3 genes were cloned in-frame with a sequence coding for GST (pEBG, Amersham Bioscience). The deletion mutants of rpS3 were designated as below. N50 represent the rpS3 mutant with the N-terminal 50 amino acid residues, C193 represent the rpS3 mutant without the N-terminal 50 amino acid residues, respectively. Each set of primers for producing cDNAs encoding the full-length and mutant rpS3 by PCR are shown in Supplemental Table S1. HEK293T cells were transfected with these recombinant plasmids using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Protein expression was confirmed by western blotting. Also, transfection of siRNA against Hsp70 or Hsp90 was performed with the transfection reagent.

2.4. In vitro pulldown assay with glutathione-S-transferase and His purification

The complete or deleted coding region of rpS3 was inserted into pGEX-5X-1 (Amersham Bioscience), respectively as previously described [30,32]. For the glutathione-S-transferase (GST) pulldown assay, full-length rpS3 or various rpS3 constructs fused to GST (GST-rpS3), GST alone, His-tagged TOT70c (cytosolic domain), or TOM20c inserted into a PET19b plasmid were expressed in Escherichia coli BL21. The expressed proteins were then purified with glutathione (GSH)-sepharose 4B beads (Amersham Pharmacia) and Ni-NTA-agarose resin (Qiagen). For the in vitro binding assay, purified His-tagged TOM proteins were incubated for 12 h at 4 °C with GST or GST-rpS3 constructs bound to beads. The beads were then washed three times in lysis buffer. The proteins were boiled in 2 x SDS sample buffer, separated by 10% SDS-PAGE, transferred to a nyloncellulose membrane, and immunoblotted using anti-His antibody.

3. Results and discussion

3.1. mtDNA damage is induced by curcumin and hydrogen peroxide

To induce mtDNA damage by cellular ROS, we treated HEK293T cells with hydrogen peroxide, changing with non-serum media including indicated concentration of hydrogen peroxide, or curcumin, compounds widely known to damage nuclear DNA and especially mtDNA [33]. As shown in Supplemental Fig. S1A, B and C, 200 μM of hydrogen peroxide increased cellular ROS levels in a time-dependent manner. Cellular ROS production was also increased in a dose-dependent manner in curcin-treated cells (Supplemental Fig. S1D, E and F). Additionally, curcumin-induced mtDNA damage was reassorted by real time PCR analysis (Supplemental Fig. S1G). We next measured the level of mtDNA damage in HEK293T cells treated with hydrogen peroxide or curcumin. According to a previous study [34], mtDNA has three regions susceptible to damage from ROS produced within their compartments. mtDNA nucleotide positions 8527–9207, 12337–14148, and 14747–15887 encode ATP synthase F0 subunit 6, NADH dehydrogenase subunit 5, and cytochrome b, respectively [35]. Among these, the most easily damaged site (8651–8850) which is critical for ATP synthesis, was used as a PCR substrate for detection of mtDNA damage. PCR assay specific for these regions (so-called “hot-regions”) has been used as a valuable method for detecting ROS-mediated mtDNA damage. The principle of this assay is that damaged DNA regions will block the progression of Taq polymerase used for the PCR reactions. As expected, the level of mtDNA PCR products significantly decreased compared to the control after treatment with hydrogen peroxide or curcumin (Fig. 1C). Furthermore, we also investigated the increased level of 8-oxoG, which is an abundant mtDNA lesion formed by ROS, in both cells treated with hydrogen peroxide and curcumin (Fig. 1D).
3.2.RpS3 is migrated to mitochondrial inner membrane by ROS-inducing agents

We next investigated the location of rpS3 protein by cytosol/mitochondria fractionation. Interestingly, rpS3 protein gradually accumulated in mitochondria as the level of cellular ROS increased (Fig. 1A, B, and Supplemental Fig. S2A). Also, we confirmed that the fractionation was purified without contamination of ER (Supplemental Fig. S2B). To exclude the possibility that hydrogen peroxide or curcumin-induced apoptosis is linked to the rpS3 accumulation, we evaluated cytochrome c release. However, we were unable to detect cytochrome c release from mitochondria into the cytosol. Furthermore, we did not detect the cleavage of caspase-3 in the concentration of curcumin below 60 μg/ml (Supplemental Fig. S3A) and nuclear DNA (nDNA) fragmentation was not induced by curcumin (Supplemental Fig. S3B, C). Therefore, we could conclude the concentration of used curcumin did not initiate apoptosis and induce nDNA damage in our condition. To further confirm the mitochondrial translocation of rpS3 protein, we performed a confocal immunofluorescent analysis using rpS3 antibody and MitoTracker. As shown in Fig. 1E and Supplemental Fig. S4, mitochondrial translocation of rpS3 was distinctly increased in hydrogen peroxide- or curcumin-treated cells compared with untreated cells. The mitochondrial translocation of rpS3 was also induced by inhibitors of oxidative phosphorylation, such as Rotenone and Antimycin A causing mitochondrial genome-specific damage (Supplemental Fig. S5).

To exclude the possibility that rpS3 would be anchored onto the mitochondrial outer membrane, the isolated mitochondrial fraction was treated with proteinase K in the presence or absence Triton X-100. As shown in Fig. 1F, protease-sensitive proteins such as ones located outside the mitochondrial outer membrane (e.g., Bcl-XL) were digested by proteinase K whereas protease-insensitive proteins such as ones inside the mitochondria (e.g., PDH) were not. When the mitochondrial fraction was co-treated with detergent such as Triton X-100, the whole mitochondrial proteins were degraded by proteinase K. Therefore, we determined that rpS3 protein is not anchored on outside the mitochondrial outer membrane. Based on these results, it seems very probable that rpS3 protein accumulated inside the mitochondria by crossing the mitochondrial outer membrane due to increased levels of cellular ROS. Recently, several lines of evidence have shown that mitochondrial repair proteins are not soluble in the matrix but interacted with the mitochondrial membrane [36,37]. To determine whether translocated rpS3 protein is localized in mitochondrial membrane particles, the mitochondrial fraction was disrupted by sonication and separated into soluble fraction (S1) and mitochondrial pellet fraction (P1) by centrifugation (Fig. 1G). mtHsp70 was used as a marker of soluble proteins in the mitochondrial matrix while the outer mitochondrial membrane protein VDAC1 was used as a marker of insoluble proteins in the mitochondrial membrane particles. As shown in Fig. 1H, although a smaller fraction of rpS3 protein was present in the S1 fraction, the majority of translocated rpS3 protein was found in the mitochondrial fraction (P1) which is an insoluble pellet portion. Also, to examine whether the location of mitochondrial rpS3 protein is inner membrane or matrix, purified mitochondria were subjected to osmotic stress with KCl buffer and then separated with inner membrane and matrix fraction through sonication and centrifugation. As shown in Fig. 1I, the final destination of mitochondrial rpS3 is located in inner membrane particle. These findings clearly showed that translocated mitochondrial rpS3 induced by ROS is loosely attached to inner mitochondrial membrane in which mtDNA repair proteins and mtDNA reside.

3.3. RpS3 is suppressed the binding with Hsp90 and strengthened the association with Hsp70 by ROS

Our previous study showed that the interaction of Hsp90 protects ribosomal proteins from ubiquitination and proteasome-dependent degradation [32]. Protein stability of rpS3 in the cytosol is regulated by interaction with Hsp90 through both the N- and C-termini of rpS3. In general, members of the Hsp90 protein family have an ATP-binding domain that plays an important role in chaperone function. Hsp90 forms homodimers in which the contact sites are localized within the C-terminus of the open conformation of the dimer. The N-terminus also comes into contact in the closed conformation of the dimer [38]. The N-terminal region of the protein contains a high-affinity ATP-binding site. Direct inhibitors of ATP binding or allosteric inhibitors of either ATP binding or ATPase activity can disrupt Hsp90 function [39]. The protein-binding region is located toward the C-terminus of Hsp90. This protein can adopt two major conformational states. The first is an open ATP-bound state and the second is a closed ADP-bound state [40]. When a natural product such as geldanamycin binds tightly to the ATP/ADP pocket of Hsp90, it inhibits the refolding of client proteins. Hsp90 is also an accessory protein critical for the migration of proteins destined for the mitochondria.

We therefore investigated whether Hsp90 or Hsp70 is indeed necessary for the mitochondrial translocation of rpS3. The protein levels of Hsp70, Hsp90 and rpS3 were unchanged in curcumin-treated cells in which cellular ROS levels increased (Fig. 2A). Co-immunoprecipitation showed that the interaction between Hsp90 and rpS3 was reduced by treatment with curcumin as ROS inducer (Fig. 2B and D) whereas association of Hsp70 and rpS3 was strengthened by curcumin (Fig. 2C and E). As shown in Fig. 2G and H, Hsp90 depletion was also reinforced the association between rpS3 and Hsp70. Taken together, these findings indicated that rpS3 associated with Hsp90 is replaced with Hsp70 by decrease of binding affinity with Hsp90. Continuously, it was examined that rpS3 is efficiently transferred from Hsp90 to Hsp70 during curcumin treatment (Fig. 2F).

To further examine the mechanism underlying rpS3 translocation into mitochondria, knockdown of endogenous Hsp70 expression with siRNA was performed (Fig. 3A). To verify whether Hsp70 functions as a guider of rpS3 leading to mitochondria, two different siRNAs against Hsp70 were transfected into HT1080 cells and then cells were incubated with curcumin for 2 h (Fig. 3A). In Fig. 3B, we assessed that the knockdown of Hsp70 prevented the mitochondrial translocation of rpS3 in curcumin treated HT1080 cells. In addition, we observed the increment of 8-oxoG level suggesting that endogenous repair activity decreased in the mitochondria of Hsp70 depleted HT1080 cells (Fig. 3C). To strengthen the notion that mitochondrial translocation of rpS3 is suppressed when the interaction between rpS3 and Hsp70 was inhibited by Hsp90 ectopically expressed GFP-Hsp90 in HT1080 cells were used (Fig. 3D). Expectedly, the mitochondrial translocation of rpS3 in Hsp90 overexpressed cells with or without curcumin was repressed more than those of GFP transfected cells or non-transfected cells (Fig. 3E, Supplemental Fig. S6). To verify whether repression of interaction between Hsp90 and rpS3 stimulates rpS3 translocation, the binding activity of Hsp90 to rpS3 was inhibited by geldanamycin (Fig. 3G and H). Interestingly, we found that rpS3 migration into the mitochondria is stimulated by each treatment of curcumin and geldanamycin whereas co-treatment of both chemicals has not an additive effect, showing that two drugs act on the same target or pathway (Fig 3I and J). Additionally, we observed that concentration of used geldanamycin did not induce ROS generation through MitoSox-ROS measurement assay (Supplemental Fig. S7). Furthermore, we showed that the 8-oxoG level in Hsp90-overexpressed cell was increased when treated with curcumin (Fig. 3F). Taken together, these findings indicated that overexpression of Hsp90 inhibits rpS3 translocation into mitochondria by suppression of association of rpS3 and Hsp70 and increase the unrepaired mtDNA damage. Moreover, our previous study showed that geldanamycin treatment facilitates the association of rpS3 with Hsp70 [32]. These results suggest that interaction with Hsp90 maintains the stability of rpS3 in the cytosol while inhibiting the migration of rpS3 protein into mitochondria or other locations such as the nucleus. When cellular ROS levels increase, the interaction of rpS3 with Hsp90 is reduced while rpS3 association with Hsp70 is increased.
3.4. N-terminus of rpS3 is recognized by TOM70 receptor

In general, protein import into the mitochondria occurs post-translationally and is regulated by molecular chaperones such as Hsp70 and Hsp90 [41–43]. Subsequently, the molecular chaperones allow proteins destined for the mitochondria to interact with translocase in the outer membrane of the TOM complex. Three subunits of this complex (Tom20, Tom22 and Tom70) function as import receptors for proteins containing an MTS [2,5,8,11,44]. To elucidate the interaction between rpS3 and TOM receptors in the cytosol, we performed a GST-
Fig. 1. Rps3 is migrated into mitochondria by ROS-inducing agents. HEK293T cells were treated with 200 μM hydrogen peroxide for the indicated time and the indicated concentrations of curcumin for 2 h. (A, B) The cells were then separated into cytosol and mitochondrial fractions. MIF, Alnexin and VDAC1 were used as markers for the cytosol, ER and mitochondrial fractions, respectively. Detection of cytochrome c in the mitochondrial fraction was used to measure mitochondrial membrane permeability. (C) Oxidative mtDNA damage was detected by PCR analysis. The 8651–8850 nucleotide positions of mtDNA, containing the coding region of ATPase 6, easily damaged by ROS were used as a substrate for detecting mtDNA damage (upper panels). The 16S ribosome region on mtDNA indicates loading control and nuclear 45S rRNA as marker of nuclear DNA for cross-contamination. (D) Each mtDNA was isolated from hydrogen peroxide or curcumin treated HEK293T cells and used in a southwestern immunoblot analysis for the detection of 8-oxoG. The amount of each mtDNA was quantified by a southern blot analysis for 16S mitochondrial rRNA gene. Nuclear 45S rRNA was used as marker of nuclear DNA for cross-contamination. (E) HT1080 cells were treated with 200 μM hydrogen peroxide for 30 min or 30 μg/ml curcumin for 2 h. The subcellular distribution of rps3 protein was observed by immunofluorescence. Live cells were stained with Mito-Tracker Red CMXRos to visualize the mitochondria in cells. The rps3 proteins were visualized by standard immunofluorescence with a fluorescein isothiocyanate-conjugated secondary antibody and appear green. The localization of rps3 in the mitochondria appears as yellow-orange in the two superimposed staining patterns (scale bar, 10 μm). (F) To identify the location of mitochondrial rps3 after mitochondria isolation and enzymatic digestion with proteinase K and co-treatment with Triton X-100. To measure proteinase K activity, PDH (a mitochondrial matrix protein) and Bcl-XL (a protein anchored to the mitochondrial outer membrane) were examined. (G) The mitochondrial fractionation scheme showing designations of two subfractions. (H) Imported rps3 protein rapidly associated with the membrane fraction. The isolated mitochondrial fractions from HEK293T cells untreated or treated with 30 μg/ml curcumin for 2 h were sonicated. Soluble and insoluble (membrane fraction) fractions were recovered by centrifugation. The indicated amounts of protein from the mitochondrial soluble and membrane fractions were loaded onto a SDS-PAGE gel at gradually increasing concentrations to determine the maximum volume without cross-contamination. The ideal protein concentration without cross-contamination between soluble fraction and membrane fraction was confirmed to be 10 μg. VDAC1 (a mitochondrial membrane protein) and mitHsp70 (mitochondrial protein found in the matrix) were used as sorting indicators for the soluble and membrane fractions. (I) Isolated mitochondria were incubated in 15 mM KCl buffer on ice for osmotic stress and then separated with inner membrane and matrix fraction by sonication and centrifugation. (Bcl-XL as outer membrane indicator, cytochrome c as inter membrane indicator, TIM22 as inner membrane indicator, PDH as matrix indicator).

pulldown assay using purified GST-rps3 protein with His-tagged Tom20cd or Tom70cd (Fig. 4A). The results of immunoblotting for proteins interacting with GST-rps3 indicated that rps3 directly interacts with Tom70cd but not Tom20cd in vitro. To verify the signal of rps3 for mitochondrial migration, we identified rps3 translocation into mitochondria with various rps3 deletion mutants (Supplemental Fig. S8A). In the results, we confirmed that N-terminus of rps3 is important region for mitochondrial translocation and 85 amino acids from N-terminus are most important region (Supplemental Fig. S8B). Also, the possibility that N-terminus of rps3 protein functions as a mitochondrial target signal (MTS) was based on MTS prediction program such as Mito-prot II and Target P. These programs predicted that possibility of RPS3 translocation into mitochondria is about 27% and 21.5%, respectively (Supplemental Fig. S8A). Also, MTS of rps3 is confirmed to be located on first 50 a.a of N-terminal and 8 amino acids were charged positively as expected (Supplemental Fig. S8C and D). To further examine whether N-terminus of rps3 is important for its mitochondrial translocation, two different GFP-tagged mutants carrying with or without N-terminus 50 amino acids were performed the mitochondrial fractionation after curcumin treatment (Fig. 4B and C). As expected, we confirmed that the rps3 deletion mutant without N-terminus did not translocate to the mitochondria. Also, we confirmed with another fusion protein, with GST-tagged deletion mutation of RPS3, (Supplemental Fig. S8A and B) also showed a very similar pattern with GFP fusion protein. In conclusion, our data suggest that the N-terminus MTS of rps3, which is unusually not cleaved in mitochondria, acts as MTS to promote the interaction with Tom70cd.

3.5. Accumulation of mitochondrial rps3 by ROS reduces 8-oxoG on mtDNA

To determine the function of rps3 accumulation in the mitochondria, we studied ectopically expressed rps3. We constructed a plasmid in which an ectopically cleavable mitochondrial targeting sequence (EMTS) was fused to the rps3 N-terminus (Fig. 5A) since exogenous rps3 without EMTS could be incorporated in cytosolic ribosomes which could affect protein translation such as mtDNA repair or ROS scavenger protein. To demonstrate that EMTS-rps3 protein was specifically expressed in the mitochondria, we performed a western blot analysis on total cell extracts (Fig. 5B) and the mitochondrial fraction after plasmid transfection (Supplemental Fig. S10). Because EMTS-rps3 is cleaved by mitochondrial processing peptidase (MPP) in mitochondrial matrix, we detected two bands in the whole cell lysate and a single band in the mitochondrial fraction. We next investigated whether ectopically expressed rps3 protein affects the accumulation of cellular ROS due to curcumin. When compared to the vector control, the level of ROS generation in curcumin treated overexpressing EMTS-rps3 was decreased from 3.5-fold to 2.5-fold (Supplemental Fig. S11) whereas not changed in the cellular ROS level in without curcumin (Supplemental Fig. S12). To assess the level of mtDNA damage by curcumin, we performed a southwestern immunoblot analysis for 8-oxoG on mtDNAs from both vector and EMTS-rps3 overexpressed cells. In Fig. 5C, the level of 8-oxoG in EMTS-rps3 over-expressed cells treated with curcumin was significantly reduced in comparison to control. Additionally, we performed a PCR method specific for the hot-region of mtDNA to analyze mtDNA...
Fig. 2. The interaction of rpS3 with Hsp90 is gradually substituted with Hsp70 by curcumin treatment. Total cell lysates (A) were extracted from HEK293T cells treated with 30 μg/ml curcumin for indicated time. To confirm the interaction of rpS3 with Hsp70, total lysate was immuno-precipitated with rpS3 (B, C, F and left panel of H), Hsp90 (D) or Hsp70 (E and right panel of H). Western blot analysis used the indicated antibodies. To confirm whether interaction of Hsp70 with rpS3 is affected by Hsp90, Hsp90 expression was depleted by two different specific siRNAs against Hsp90 (G) and immunoblot and immunoprecipitation analysis were performed with indicated antibody.

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4. Conclusions

RpS3 is a component of the 40S ribosome small subunit and plays a critical role in the initiation of protein translation [45]. Many ribosomal proteins have extra-ribosomal functions [46]. RpS3 also serves as a DNA repair endonuclease/lyase that participates in repairing DNA damaged by genotoxins including UV, hydrogen peroxide and parquet. In a previous study, we found that rpS3 protein has a lyase activity against oxidative-damaged and UV-irradiated DNA containing AP sites, thymine glycol, 8-oxoguanine, etc. [18,19] and results in decreased cellular ROS generation.

dOgg1 and is involved in the repair pathway of mtDNA damaged by hydrogen peroxide in Drosophila [47].

In the present study, we showed that protein levels of other ribosomal protein such as rpS6, rpS11 and rpL13 in mitochondria (Supplemental Fig. S13) were not changed by curcumin but, rpS3 was translocated into the mitochondria when the cell was exposed to reagents such as hydrogen peroxide and curcumin. However, many studies have demonstrated that the organellar homolog of rpS3 is clearly absent from the mitochondrial genomes of mammalian cells [48]. Recent mitochondrial proteomic studies have further confirmed the existence of rpS3 in the mitochondria but not as a component of the mitochondrial 28S subunit [48]. We concluded that the translocated rpS3 is involved in the repair of mtDNA damage caused by ROS.

Most mitochondrial proteins are imported by TOM complex that helps form surface receptors which recognize proteins destined for the mitochondria [2–5, 7]. Mitochondrial targeting requires various cytosolic factors such as the molecular chaperones Hsp70 and Hsp90 [5, 41–43]. Complexes containing Hsp70/Hsp90 guide the mitochondrial proteins to Tom70 mitochondria receptors which contain a clamp-type tetratricopeptide repeat (TPR) domain [5, 43]. Our data demonstrated that rpS3 directly interacts with the cytoplasmic domain of Tom70 (Tom70cd) but not the cytoplasmic domain of Tom20 (Tom20cd). Further deletion analysis identified 1–50 amino acids of rpS3 as a dominant MTS region containing a lysine at positions 7, 8, 10 and arginine residues at positions 9, 27, 40, 45. According to our previous study [32], the
stability of cytosolic rpS3 protein is regulated by interaction with Hsp90 via both the N- and C-termini. Therefore, we hypothesized that Hsp90 can bind upon the MTS of rpS3 in the cytosol. We also found that interaction between rpS3 and Hsp90 was reduced by treatment with curcumin before rpS3 protein is transferred to Hsp70 (Fig. 2). These results exactly coincide with our previous data showing that geldanamycin treatment increases the dissociation of rpS3 from Hsp90 while increasing its association with Hsp70. Taken together, Hsp90-rpS3 interaction appears to prevent MTS exposure to the Tom70 protein on the mitochondrial outer membrane. Subsequently, Hsp70-rpS3 interaction induced by cellular ROS facilitates the access to the Tom70 protein. It should be noted that the interaction between rpS3 and Hsp90 also inhibits the phosphorylation by PKCδ which enables rpS3 to migrate into the nucleus to promote DNA repair [32].

Fig. 3. The repression of mitochondrial translocation of rpS3 increases 8-oxoG on mtDNA which is induced by curcumin. To confirm whether interaction of Hsp70 with rpS3 contributes to mitochondrial translocation of rpS3, depletion of Hsp70 was performed by two different specific siRNAs (A), and treated with 30 μg/ml curcumin for 2 h (A, B and C) and then mitochondria were purified to confirm mitochondrial rpS3 (B) and 8-oxoG (C). Lamin A/C or Fibrillarin were used as nuclear markers to exclude contamination in purified mitochondrial fractions. HEK293T cells were transfected with GFP-Hsp90 or GFP only expressed-plasmid DNA and then treated with 30 μg/ml curcumin for 2 h (D, E and F) or 1.5 μM geldanamycin for 4 h (G–J). Immunoprecipitation was performed with anti-rpS3 Ab (H). Antibodies were subjected to Western blot analysis using the indicated antibodies. (I) HEK293T cells were treated with or without 30 μg/ml curcumin for 2 h after pre-treatment with 1.5 μM geldanamycin for 4 h. The mitochondrial fractions were isolated as described in "Materials and Methods" and immunoblotted with anti-rpS3, anti-PDH (as a mitochondria loading control), and anti-MIF (as a cytosolic protein marker) antibodies. (J) For quantitative analysis, the intensity of rpS3 band on immunoblot analysis was normalized to the intensity of an each PDH band. Error bars represent the SD of the mean of at least three independent experiments.
Fig. 4. N-terminus of rpS3 is recognized by TOM70 receptor. His-Tom20cd, Tom70cd, and GST-rpS3 fusion proteins were expressed in E. coli, and purified with Ni-NTA-agarose resin or glutathione (GSH)-sepharose 4B beads. (A) To measure fusion protein expressions, the purified proteins were separated by SDS-PAGE and stained with Coomassie Blue or performed Immuno-blotting with anti-His antibody (input). Purified His-Tom20cd and His-Tom70cd were incubated with GST and GST-conjugated rpS3. After recovery by GST-pulldown, the remnant proteins in the pellet were separated by SDS-PAGE and subjected to an immunoblot assay with anti-His antibody (GST-pulldown). (B) Schematic figures of GFP-rpS3 deletion mutants. (C) GFP-rpS3 derivatives were used for identifying the mitochondrial fraction. Each GST-rpS3 construct was transfected into HEK293T cells. After 24 h, the cells were treated with 30 μg/ml curcumin for 2 h. The expression of GFP-rpS3 derivatives was assessed by immunoblot analysis. Mitochondrial fractions were isolated from HEK293T cells transfected with each one of the GFP-rpS3 constructs and immunoblotted with the indicated antibodies. MIF (a cytoplasmic protein); PDH (a mitochondrial loading control).

Fig. 5. Accumulation of mitochondrial rpS3 by ROS reduces 8-oxoG on mtDNA. (A) rpS3 constructs with EMTS were over-expressed in mitochondria through a pCMV/myc/mito plasmid. The EMTS was attached to the N-terminus of full-length rpS3. Additionally, C-terminus of the protein was tagged with c-Myc (Long form). The EMTS was cleaved upon entry into the mitochondria (Cleaved form). (B) HEK293T cells were transfected with pCMV/Myc/mito (vector) or pCMV/Myc/mito-rpS3 (rpS3-Myc). Total cell lysates were subjected to immunoblot analysis with anti-c-Myc and anti-rpS3 antibodies. Exogenous rpS3 protein appeared as two bands. The upper band is a cytosolic long form (indicated with an “L”) and the lower band is a mitochondrial cleaved form (indicated with a “C”). (C, D) Vector or rpS3-Myc (EMTS-S3) transfected HEK293T cells were treated with the curcumin or H2O2 and purified mitochondrial DNA for Southwestern immunoblot analysis with anti-8-oxoG antibody (C). PCR analysis was then performed to detect oxidative mtDNA damage (D).
Here, through the detection of 8-oxoG adducts in mtDNA, we showed that mitochondrial translocation of rpS3 protein results in the reduction of mtDNA damage generated by curcumin or hydrogen peroxide treatment. There are many conflicting opinions about the repair activity of rpS3 against oxidative DNA damage. Ko et al. reported that rpS3 protein can activate uracil-DNA glycosylase activity upon binding [49]. Hegde et al. also showed that hOGG1 activity is increased by interaction with rpS3 [17,50]. However, the same group presented an opposite view stating that rpS3 overexpression causes the accumulation of DNA damage in response to oxidative stress [51]. In addition to this, we investigated whether EMTS-tagged rpS3 contributes to the increase of DNA repair activity of mammalian rpS3 [52]. In our previous report [19], it was identified that yeast Rps3 possesses key residues at Asp154 and Lys200 corresponding to mammalian rpS3 Asp154 and Lys202 for mtDNA repair function. So, we made MTS tagged-rpS3 point mutants (D154A, K202N and DAKN as double mutant of both D154A and K202N) and investigated the levels of mtDNA repair activity of these mutants. Interestingly, only the cells expressing double-mutant were highly sensitive to mtDNA damage by curcumin (Supplemental Fig. S16). Moreover, we identified that rpS3 were co-localized with APE1/Ref-1 or DNA Pol. γ in mitochondria, suggesting that mitochondrial rpS3 interacts with mtBER proteins (Supplemental Fig. S17A and B). But, we should further investigate how mitochondrial rpS3 recognizes mtDNA damage, repairs damaged mtDNA. According to reports, ribosomal protein S3 (rpS3) has been known to possess a multifunction in nucleus such as a subunit of NF-κB complex that influences on specific gene transcription [27] and a binding partner of HDM2 that stabilizes p53 stability [28]. And, rpS3 phosphorylation by PKCδ or IKKδ is responsible for its nuclear localization [15]. However, we did not confirm the modification in mitochondrial rpS3. Therefore, we supposed that there is different conformational modification exposing the MTS.

In recent years, studies have performed to examine the relationship between mtDNA mutations and human diseases such as hereditary diseases and cancers [53,54]. Interestingly, it was also reported that the expression of Hsp90 protein in colorectal cancers is increased and that the Hsp90 inhibitor 17AAG (17-allylamino,17-demethoxygeldanamycin) can be administered to patients as an anti-cancer therapy [55]. Based on these findings, we propose that rpS3 translocation into the mitochondria in response to cellular ROS is sufficient to repair mtDNA damage in normal cells. Therefore, we propose a model for the mitochondrial function of rpS3 in the presence of increased cellular ROS levels (Fig. 6). First, cytosolic rpS3 interacts with Hsp90 to protect the rpS3 protein from proteasomal degradation. Second, increased ROS levels induce the transfer of rpS3 from Hsp90 to Hsp70 and the exposure of MTS by dissociation of Hsp90 on N-terminus of rpS3 for mitochondrial localization. Third, the interaction between rpS3 and Hsp70 facilitates rpS3 translocation into the mitochondria via Tom70 recognizing the MTS. Finally, mitochondrial rpS3 repairs ROS-induced mtDNA damage through its endonuclease activity, resulting in the decrease of cellular ROS generation. On the other hand, rpS3...
phosphorylated by PKCαs also can be translocated into the nucleus to re-pair nuclear DNA [15].

Acknowledgement

We would like to thank Nikolaus Pfanner for providing Tom20cd and Tom70cd recombinant plasmid DNA. This work was supported in part by NRF-2012R1A2A1A01009027 grant and Korea University grant. Kim HD was supported by Korea University Fellowship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.07.015.

References


