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Human G-proteins, ObgH1 and Mtg1, associate with the large mitochondrial ribosome subunit and are involved in translation and assembly of respiratory complexes

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ABSTRACT

The bacterial homologues of ObgH1 and Mtg1, ObgE and RbgA, respectively, have been suggested to be involved in the assembly of large ribosomal subunits. We sought to elucidate the functions of ObgH1 and Mtg1 in ribosome biogenesis in human mitochondria. ObgH1 and Mtg1 are localized in mitochondria in association with the inner membrane, and are exposed on the matrix side. Mtg1 and ObgH1 specifically associate with the large subunit of the mitochondrial ribosome in GTP-dependent manner. The large ribosomal subunit stimulated the GTPase activity of Mtg1, whereas only the intrinsic GTPase activity was detectable with ObgH1. The knockdown of Mtg1 decreased the overall mitochondrial translation activity, and caused defects in the formation of respiratory complexes. On the other hand, the depletion of ObgH1 led to the specific activation of the translation of subunits of Complex V, and disrupted its proper formation. Our results suggested that Mtg1 and ObgH1 function with the large subunit of the mitochondrial ribosome, and are also involved in both the translation and assembly of respiratory complexes. The fine coordination of ribosome assembly, translation and respiratory complex formation in mammalian mitochondria is affirmed.

INTRODUCTION

Mitochondria possess their own genome, mitochondrial DNA (mtDNA). The mammalian mitochondrial DNA encodes 2 rRNAs, 22 tRNAs and 13 proteins that are subunits of the respiratory chains (1). To synthesize these 13 proteins, mitochondria utilize their own protein synthesis machinery and ribosomes. In mammalian mitochondria, the ribosome is a 55S particle composed of a small 28S subunit and a large 39S subunit. The 55S ribosome contains two rRNAs and ~80 ribosomal proteins (2). All mitochondrial ribosomal proteins are encoded by genomic DNA, which are synthesized in the cytosol and imported to mitochondria. These ribosomal proteins assemble with mitochondrial rRNA, transcribed from mtDNA, to form the mitochondrial ribosome. Mitochondrial ribosomes are known to interact with the inner membrane (3), to ensure the co-translational insertion of nascent proteins within the inner membrane. Recently, it has been reported that mutations in mitochondrial ribosomal proteins cause neurodegenerative diseases (4–9), and some of these diseases may be consequences of defects in mitochondrial ribosome assembly (4,7–9). To elucidate the pathogenic mechanisms of these diseases, it is important to understand the mechanism of mitochondrial ribosome assembly.

Little is known about biogenesis of mitochondrial ribosomes. So far, three G-proteins are known to function in mitochondrial ribosome assembly in yeast: the mitochondrial GTPases 1, 2 and 3 (Mtg1, Mtg2 and Mtg3). Mtg1 and Mtg2 function in the assembly of the large subunit (10,11), whereas Mtg3 is involved in the assembly of the small subunit (12). In mammalian mitochondria, mtRsfA/C7orf30, is involved in the assembly of the large subunit (13,14). Two G-proteins, Era-like 1 (ERAL1) and Nitric oxide associated-1 (Noa1/C4orf14) (a homologue of yeast Mtg3), are involved in the assembly of the small subunit (15–17). In the present study, we analyzed human Mtg1 and ObgH1, the homologues of yeast Mtg1 and Mtg2, respectively.

ObgH1, also known as GTP-binding protein 5 (GTPBP5), belongs to the Obg family. The Obg family

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proteins are P-loop GTPases found in bacteria and eukaryotes (18). The *Escherichia coli* Obg protein (ObgE) binds to the large ribosomal subunit (19,20). In yeast, Mtg2p, the Obg protein homologue, is localized to the mitochondria and also binds to the large ribosomal subunit (11). The dysfunction of ObgE leads to a reduction in the levels of 70S ribosomes, an increase in the levels of the 30S and 50S subunits, and an accumulation of 50S subunit assembly intermediates (19–21). Moreover, ObgE mutations result in rRNA processing defects and the accumulation of 16S and 23S rRNA precursors (19–21). In the 50S assembly intermediates, the levels of ribosomal proteins L16, L33 and L34 are reduced (20). As L16, L33 and L34 are late assembly proteins, ObgE is suggested to be involved in the later stages of 50S subunit assembly (20,21). ObgH1 expression rescued the effects of ObgE-depletion in *E. coli* (22), suggesting that ObgH1 is able to function in ribosome maturation.

Mtg1, which is also known as GTP-binding protein 7 (GTPBP7), is a YlqF/YawG family protein. In *Bacillus subtilis*, the depletion of a YlqF/YawG family protein, RbgA, causes a reduction in 70S ribosomes and the accumulation of the 45S assembly intermediate of the 50S subunit (23–26). The 45S intermediate lacks the ribosomal proteins L16, L27 and L36. RbgA is associated with both the 45S intermediate and 50S subunit, but its interaction with the 50S subunit requires the presence of GTPγS (23). The GTPase activity of RbgA is stimulated by the 50S subunit, but not the 45S intermediate (27). These results suggested that RbgA recruits the ribosomal proteins L16, L27 and L36 at a late assembly step (27,28). In yeast mitochondria, Mtg1 mutations result in respiratory deficiency because of a defect in mitochondrial translation (10). Mutations of the large subunit rRNA can suppress the mitochondrial translation defect of mtg1 null mutants (10). Therefore, Mtg1 is suggested to be involved in the assembly of mitochondrial large ribosomal subunits. As human Mtg1 is able to rescue the respiratory deficiency of a yeast mtg1 mutant, human Mtg1 is considered to be involved in mitochondrial ribosome assembly (10).

The assembly of ribosomes requires the processing and modification of rRNAs, coordinated with the temporal association of ribosomal proteins. This process is regulated by assembly factors, such as helicases, modifying enzymes and GTPases. In the present study, we sought to elucidate the functions of two human G-proteins, ObgH1 and Mtg1, in the biogenesis of ribosomes in human mitochondria.

MATERIALS AND METHODS

Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium (high glucose) (Wako), supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37°C in a 5% CO_2 atmosphere.

Isolation of mitochondria from human cells and mitochondrial fractionation

Mitochondria were isolated according to the previously published procedure (29). For mitochondrial fractionation, purified mitochondria were swollen by the addition of swelling buffer [10 mM KH_2PO_4 (pH 7.4)] at a concentration of 1000 μg of mitochondrial protein per milliliter and incubated for 20 min at 4°C with gentle mixing. An equal volume of shrinking buffer [10 mM KH_2PO_4 (pH 7.4), 32% (w/v) sucrose, 30% (v/v) glycerol and 10 mM $MgCl_2$] was added, and the samples were incubated for an additional 20 min at 4°C. The suspension was centrifuged at 10 000*g* for 30 min. The pellet was composed of mitoplasts, and the supernatant was composed of the outer membrane and components of the intermembrane space. The supernatant was centrifuged at 150 000*g* for 1 h, using an SW41Ti rotor (Beckman Coulter). The pellet was rinsed with MSED[−] buffer [20 mM HEPES–KOH (pH 7.4) 225 mM mannitol, 75 mM sucrose and 1 mM ethylenediaminetetraacetic acid] and was centrifuged at 150 000*g* for 1 h, using an SW41Ti rotor (Beckman Coulter). The pellet was composed of the outer membrane, and the supernatant was composed of the intermembrane space components. The pellet was suspended in RIPA buffer [25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% nonidet P-40 (NP-40), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid and protease inhibitor (Roche)]. Triton X-100 was added to the supernatant to a final concentration of 0.1% (v/v), and the sample was enriched. The mitoplasts were washed twice with MSED[−] buffer and resuspended in MSED[−] buffer. The mitoplast solution was disrupted by sonication and then centrifuged at 150 000*g* for 1 h, using a TLA100.2 rotor (Beckman Coulter). The supernatant was composed of the matrix, and the pellet included the inner membrane. The pellet was washed with MSED[−] buffer and suspended in RIPA buffer.

Proteinase K assay

Mitochondria and mitoplasts were suspended in MSED[−] buffer. Proteinase K (TAKARA) was added to final concentrations of 0, 0.2 and 2 μg/ml. These samples were incubated on ice for 30 min. The reactions were stopped by the addition of phenylmethylsulfonyl fluoride (PMSF) and LiDS sample buffer [40 mM Tris-HCl (pH 6.8), 2% (w/v) lithium dodecyl sulfate (LiDS), 10% (v/v) glycerol, 10% (w/v) bromophenol blue and 350 mM β-mercaptoethanol]. The samples were heated at 95°C for 5 min, placed on ice and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) followed by detection by immunoblotting.

Expression and purification of ObgH1 and Mtg1

ObgH1/pET15b and Mtg1/pET15b, the *E. coli* expression vectors for N-terminal histidine-tagged human ObgH1 and human Mtg1, respectively, were constructed as follows. The DNA fragment encoding ObgH1 (amino acids 31 through 406) was obtained by polymerase chain reaction using ObgH1/pGEX5X-3 (22), and that encoding Mtg1 (amino acids 27 through 334) was obtained by reverse transcription–polymerase chain reaction, using poly(A) RNA from HeLa cells. Each fragment was cloned into pET15b (Novagen).

ObgH1/pET15b and Mtg1/pET15b were transformed into *E. coli* Rosetta (DE3)/pLysS cells (Novagen). Protein expression was induced with 0.1 mM isopropyl-1-thio-D-galactopyranoside at 18°C overnight. The cells were resuspended in lysis buffer [50 mM Tris–HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 3.5 mM β-mercaptoethanol, 0.1 mM PMSF and 20 μM GDP] and lysed by sonication. After the centrifugation at 140 000*g* for 2 h, the supernatant was collected. The proteins were adsorbed to Ni-NTA resin (QIAGEN), and the resin was washed with Ni-NTA wash buffer [50 mM Tris–HCl (pH 7.5), 1 M NH_4Cl, 20 mM imidazole, 10% glycerol, 3.5 mM β-mercaptoethanol and 20 μM GDP]. The proteins were eluted with Ni-NTA elute buffer [50 mM Tris–HCl (pH 7.5), 100 mM KCl, 150 mM imidazole, 10% glycerol, 3.5 mM β-mercaptoethanol and 20 μM GDP] and dialyzed against dialysis buffer [20 mM HEPES–KOH (pH 7.5), 100 mM KCl, 10% glycerol, 20 μM GDP and 7 mM β-mercaptoethanol]. The proteins were further purified by HiTrap SP HP column chromatography (GE Healthcare), using the buffer [20 mM HEPES–KOH (pH 7.5), 100–300 mM KCl, 10% glycerol, 20 μM GDP and 7 mM β-mercaptoethanol]. The proteins were dialyzed against stock buffer [20 mM HEPES–KOH (pH 7.5), 200 mM KCl, 10% glycerol, 20 μM GDP and 7 mM β-mercaptoethanol] and were concentrated to 5 mg/ml. ObgH1 and Mtg1 were purified to 90 and 100% homogeneity, respectively.

Preparation of ribosomes

We used mitochondrial 55S ribosomes from pig liver mitochondria, as a model for human mitochondria, because pig mitochondrial ribosomes share high homology with those from human mitochondria and are easier to prepare. The mitochondrial 55S ribosomes, and the 28S and 39S subunits, were prepared from pig liver mitochondria according to the previous report (30,31). Briefly, the mitoplasts were solubilized with Triton X-100 and centrifuged at 21 000*g* for 30 min. The supernatant (S30) was further centrifuged at 44 000*g* for 16 h to obtain the supernatant (S100) and the pellet (crude ribosomes). The crude ribosomes were layered on 10 and 30% sucrose gradient (at 20 and 2 mM $MgCl_2$, for 55S and the subunits, respectively), and the objective ribosomal subunits were recovered. The *E. coli* 70S ribosomes, and the 30S and 50S subunits, were prepared as described previously (32).

Ribosome-binding assay

Reactions were performed in 100 μl of binding buffer [15 mM Tris–HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 1 mM guanine nucleotide] containing 0.2 μM 55S ribosome and 2 μM ObgH1/Mtg1. After a 20 min incubation at room temperature, the reaction mixtures were fractionated on 15–30% (w/v) sucrose gradients in binding buffer, by centrifugation at 39 000*g* for 5.5 h using an SW41Ti rotor (Beckman Coulter). The gradients were recovered from the top to the bottom, using density gradient fractionators (Towa Labo, Model 152-001) while monitoring the absorbance at 260 nm. The fractions were separated by SDS–PAGE and subjected to immunoblot analyses. For the analysis of the interactions of the endogenous ObgH1 and Mtg1 with mitochondrial ribosomes in mitochondrial lysates, isolated mitochondria were extracted with buffer [10 mM Tris–HCl (pH 7.5), 100 mM NH_4Cl, 10 mM $Mg(OAc)_2$, 2% NP-40, 7 mM β-mercaptoethanol, 1 mM PMSF and 80 U/ml Recombinant RNase Inhibitor (TAKARA)] in the absence of guanine nucleotides and in the presence of GDPNP, in the cases of ObgH1 and Mtg1, respectively. The extracts were centrifuged at 40 000*g* for 10 min, and the supernatants were fractionated on 10–30% sucrose gradients. The fractions were separated by SDS–PAGE and subjected to immunoblot analyses.

Double-stranded RNA transfection

The following double-stranded RNAs (dsRNAs) were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions: dsRNAs for ObgH1: sense 5′-GCAACGGUGGACAC GUCAU-3′ and antisense 5′-AUGACGUGUCCACCGUU GCCU-3′; for Mtg1: sense 5′-GUUA AAGGGCUGUUU CUU-3′ and antisense 5′-AAGAAACAGGCCCUUUAA CUG-3′ and for control (Firefly Luciferase): sense 5′-GU GCGCUGCUGGUGCCAAC-3′ and antisense 5′-GUU GG CACCAGCAGCGCACUU-3′). For the analyses of the levels of mitochondrial ribosomal proteins and the formation of respiratory chain complexes, the cells were transfected again after 3 days. At 5 days after the first transfection, the cells were harvested. For the mitochondrial translation assay and the northern analysis, the cells were transfected again after 4 days. At 7 days after the first transfection, the cells were used for the mitochondrial translation assay and RNA isolation.

Mitochondrial translation

The translation of proteins encoded by mtDNA was assayed by [^{35}S] methionine labeling for 2 h, in the presence of emetine, an inhibitor of cytoplasmic translation. After labeling, the cells were harvested, resuspended in phosphate-buffered saline (PBS) containing 1% [v/v] Triton X-100 and centrifuged to remove insoluble materials. The supernatant was separated by SDS–PAGE on a 15% gel, and the radioactive bands were visualized with a BAS5000 bio-imaging analyzer, using an Imaging Plate (Fujifilm).

RNA isolation and northern blotting

Total RNA from HeLa cells was isolated using ISOGEN (NIPPON GENE), according to the manufacturer's instructions. For northern blots, RNA was electrophoresed on 1% agarose gels under denaturing conditions and transferred to Hybond-NX membranes (GE Healthcare). Radiolabeled probes were generated by phosphorylating the oligo DNAs (5′-CTTCTATTGACTTGGGTTAATC GTGTGACC-3′ for 12S mt rRNA and 5′-ATCTTGGAC AACCAGCTATCACCAGGCTCG-3′ for 16S mt rRNA).

Blue Native (BN) PAGE

HeLa cells were harvested, resuspended in PBS to a final protein concentration of 5 mg/ml and mixed with an equal volume of 4 mg/ml of digitonin in PBS. After an incubation for 15 min on ice with occasional mixing, the sample was centrifuged for 10 min at 10 000*g* at 4°C. The mitochondria-enriched pellet was washed with PBS twice. The pellet was solubilized for 15 min on ice with 1% digitonin in Solubilization buffer (50 mM NaCl, 5 mM 6-aminohexanoic acid, 50 mM imidazole and 10% glycerol), centrifuged at 20 000*g* for 20 min and subjected to Blue-Native gel electrophoresis on a NativePAGE Novex 3–12% Bis–Tris Gel (Invitrogen). After an incubation at 60°C for 15 min in denaturing buffer [20 mM Tris–HCl (pH 6.8), 1% SDS and 100 mM β-mercaptoethanol], the proteins were electrically transferred to PVDF membranes and analyzed by immunoblotting.

Measurement of GTPase activity

The ribosome-dependent GTPase activity of Mtg1 was measured as described previously (33,34). Briefly, assays were performed in 25 µl of GTPase assay buffer [20 mM HEPES–KOH (pH 7.5), 100 mM KCl, 4.5 mM $Mg(OAc)_2$, 1 mM DTT, 2 mM spermidine and 0.05 mM spermine], containing 0.2 µM 55S ribosomes and 10 µM of Mtg1. Reactions were started by adding 0.5 µl of γ-[^{32}P]GTP (7.5 mM, ~50 cpm/pmol). After 20 min incubation at 30°C, the reactions were terminated by adding 100 µl of 0.1 N H_2SO_4–1.5 mM NaH_2PO_4 and 25 µl of 5% sodium molybdate. Phosphomolybdate complexes were extracted with 250 µl *n*-butanol. The radioactivity within 200-µl aliquots of the butanol layer was counted with a scintillation counter.

Antibodies

The anti-ObgH1, anti-Mtg1, anti-MRPL7/L12 and anti-MRPS12 antibodies were produced in our laboratory. The anti-Complex I 39 kDa subunit, anti-Complex II 70 kDa subunit, anti-Complex III subunit Core 1, anti-Complex IV subunit I, anti-Complex IV subunit II, anti-Complex IV subunit IV and anti-Complex V a subunit antibodies were obtained from Molecular Probes. The anti-Porin antibody was obtained from Calbiochem. The anti-cyt C antibody was obtained from BioVision. The anti-Hsp60 antibody was purchased from Sigma. The anti-Tom40 and anti-ATP8 antibodies were purchased from Santa Cruz Biotechnology. The anti-Tom20 and anti-MRPS29 antibodies were obtained from BD Bioscience. The anti-MRPL45 antibody was obtained from Abcam. The anti-EF-Tu/Tsmt antibody was a gift from Prof. Linda Spremulli, University of North Carolina.

RESULTS

Mitochondrial localization of ObgH1 and Mtg1

Previous immunofluorescence studies revealed that ObgH1 and Mtg1 associate with mitochondria in human cells (10,11). We initially confirmed the mitochondrial localization of ObgH1 and Mtg1 by mitochondrial fractionation. Mitochondria were separated into outer membrane, intermembrane space, inner membrane and matrix fractions. ObgH1 and Mtg1 were detected in the inner membrane fraction, indicating that both are associated with the mitochondrial inner membrane (Figure 1A). Proteinase K assays also confirmed the mitochondrial localization of the proteins. ObgH1 and Mtg1 were protected against 0.2 µg/ml of proteinase K in both intact mitochondria and mitoplasts (Figure 1B). Hsp60, a matrix protein control, was protected against proteinase K in both mitochondria and mitoplasts. Tom40, an outer membrane protein control exposed to the intermembrane space, was digested by proteinase K in mitoplasts. These results indicated that ObgH1 and Mtg1 are localized within mitochondria, in association with the inner membrane, and are exposed on the matrix side.

Interactions of ObgH1 and Mtg1 with the large mitochondrial ribosome subunit

ObgH1 and Mtg1 belong to the Obg family and YlqF/YawG family proteins, respectively. The proteins from both families reportedly associate with the large subunit of the ribosome (11,19,20,23,35) in a GTP-dependent manner (20,23,27). The hydrolysis of GTP is thought to stimulate the dissociation of the proteins from the ribosome.

We examined the interactions of ObgH1 and Mtg1 with mitochondrial ribosomes *in vitro*, using the recombinant proteins (Figure 2A) and a mitochondrial extract (Figure 2B). We used mitochondrial ribosomes purified from pig liver, for convenience, in the *in vitro* interaction assays. In Figure 2A, the purified mitochondrial ribosomes and the recombinant ObgH1 or Mtg1 protein were incubated in the presence of various guanine nucleotides. The mixtures were then fractionated on a sucrose density gradient, and ObgH1 and Mtg1 were detected by immunoblotting of the fractions. In Figure 2B, the mitochondrial extracts were fractionated on a sucrose density gradient, and the fractions were analyzed by immunoblotting against ObgH1 and Mtg1. A substantial amount of endogenous GTP is present in the mitochondrial extract. In both cases, ObgH1 and Mtg1 co-fractionated with the 39S subunit, the large subunit of the mitochondrial ribosome, and no significant associations of the proteins with the 55S ribosome were observed.

It is notable that the binding of Mtg1 to the 39S subunit was detectable only in the presence of GDPNP, a non-hydrolyzable analogue of GTP (Figure 2A right and B right and Supplementary Figure S1). This observation is consistent with the fact that the bacterial YlqF/YawG proteins show higher affinity to ribosomes in the presence of GDPNP than GTP (23,27), and they may explain why the association of yeast Mtg1 with the ribosome has not been detected in the absence of guanine nucleotides (10). The result that the binding of Mtg1 to the 39S subunit was not detectable in the presence of GTP suggested that Mtg1 dissociated from the 39S subunit after GTP hydrolysis.

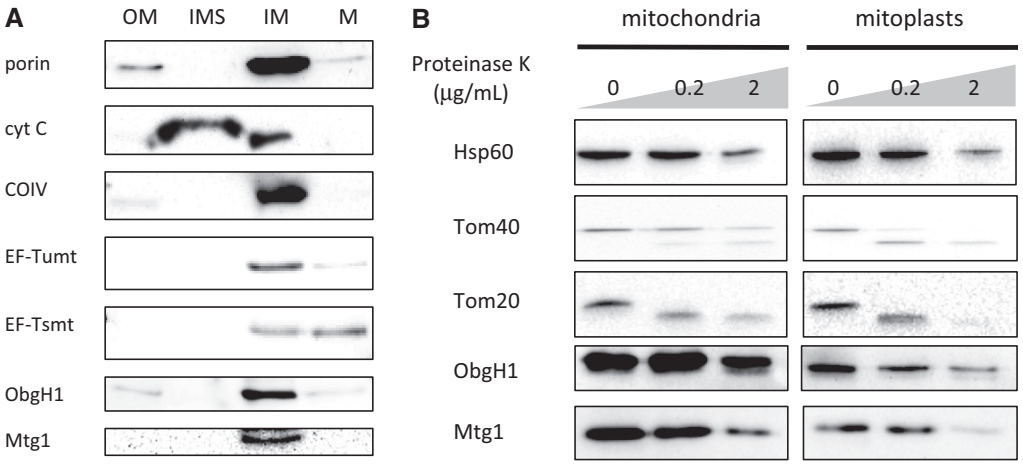

Figure 1. Submitochondrial localization of ObgH1 and Mtg1. (**A**) Mitochondria from HeLa cells were separated into outer membrane (OM), intermembrane space (IMS), inner membrane (IM) and matrix (M) fractions, and the proteins were subjected to immunoblot analyses. Porin (VDAC1), an outer membrane protein; cyt C, an intermembrane space protein; COIV, an inner membrane protein; EF-Tumt and EF-Tsmt, matrix proteins. (**B**) Mitochondria and mitoplasts were treated with proteinase K and subjected to immunoblot analyses. Hsp60, a matrix protein; Tom40, an outer membrane protein exposed to the intermembrane space; Tom20, an outer membrane protein exposed to the cytosol.

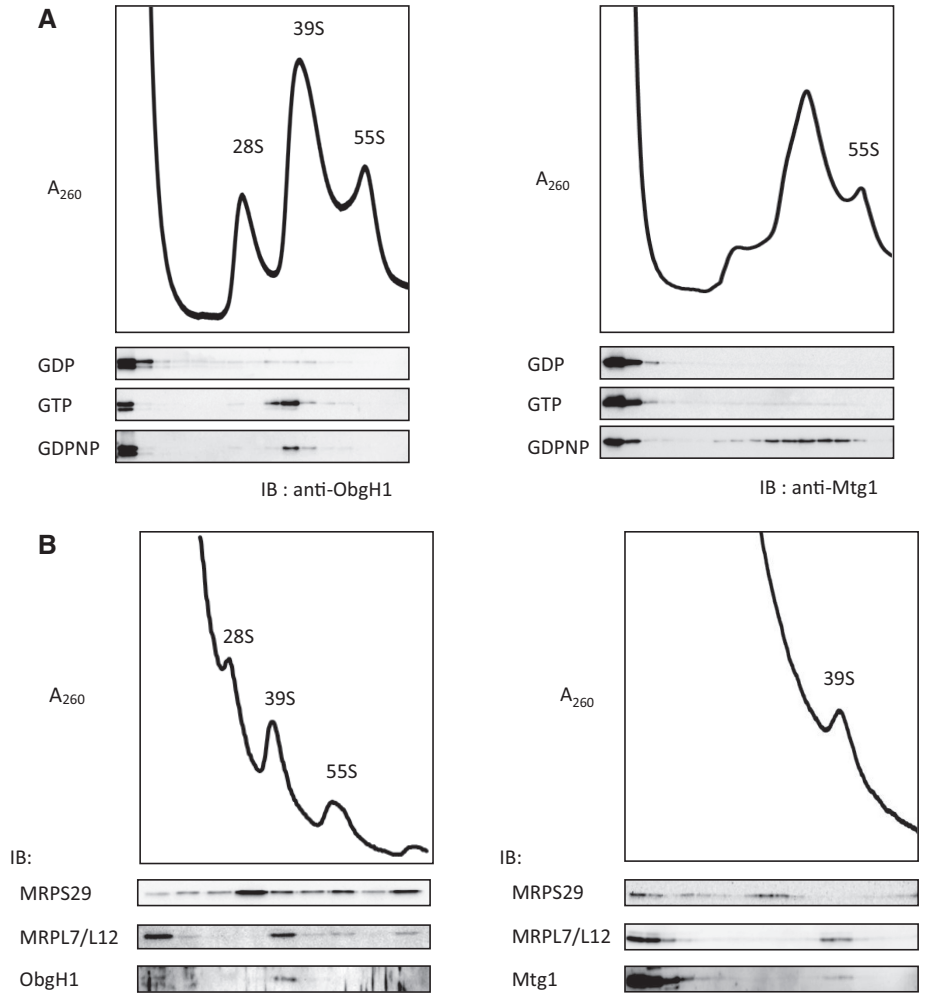

Figure 2. Interactions of ObgH1 and Mtg1 with mitochondrial ribosomes. (**A**) Recombinant ObgH1 (left panels) and Mtg1 (right panels) (f.c. 2 μM) were incubated with mitochondrial 55S ribosomes (f.c. 0.2 μM) in the presence of various guanine nucleotides and subjected to sucrose density gradient centrifugation. Gradient fractions were analyzed by immunoblotting with the indicated antibodies. (**B**) Mitochondrial extracts were separated on the sucrose gradients, and the fractions were subjected to immunoblot analyses for ObgH1 (left) and Mtg1 (right). The mitochondrial extract was prepared in the absence of guanine nucleotides and in the presence of GDPNP (a non-hydrolyzable analogue of GTP), in the cases of ObgH1 and Mtg1, respectively.

Depletion of ObgH1 and Mtg1 results in defects of mitochondrial translation

The Obg and YlqF/YawG proteins were shown in bacteria to associate with the large subunit of the ribosome, and they are involved in the late stage of ribosome assembly (20,23,25,26). We analyzed the effects of ObgH1 and Mtg1 gene silencing, to assess the roles of human ObgH1 and Mtg1 in mitochondrial ribosome assembly. After treating HeLa cells for 5 days with dsRNA, the levels of the ObgH1 and Mtg1 proteins were repressed to ~15% and 10%, respectively (Figure 3B).

We observed the steady-state levels of the mitochondrial ribosomal RNA by northern blotting, after 7 days of dsRNA treatment (Figure 3A). The levels of mitochondrial rRNAs (12S and 16S) were not affected, and the precursors of the rRNAs were not detected. These results indicated that neither ObgH1 nor Mtg1 is involved in the transcription and processing of mitochondrial rRNAs, in contrast to the bacterial homologues (19,20). The steady-state levels of the mitochondrial ribosomal proteins were analyzed by immunoblotting, after 5 days of dsRNA treatment (Figure 3B). No alterations in the levels of the examined mitochondrial ribosomal proteins were detected (L7/L12, L45, S12 and S29). We also investigated the mitochondrial protein synthesis activity after 7 days of dsRNA treatment (Figure 3C). HeLa cells were pulse-labeled with [^{35}S] methionine in the presence of emetine, an inhibitor of cytoplasmic translation. We found that the ObgH1 knockdown changed the pattern of mitochondrial translation. The synthesis of most proteins decreased, whereas the synthesis of ATP6 and ATP8 specifically increased, by ~2-fold. On the other hand, the Mtg1 knockdown decreased the overall mitochondrial translation rate to ~60%.

At present, it is not clear whether ObgH1 and Mtg1 are involved in mitochondrial ribosome assembly, as such a defect could occur without affecting the steady-state levels of the ribosomal proteins (14). However, our results clearly suggested that ObgH1 and Mtg1 affect the activity of the mitochondrial ribosome, and they are involved in mitochondrial translation.

Depletion of ObgH1 and Mtg1 leads to defective respiratory chain complex assembly

All of the proteins synthesized in mitochondria are the subunits of respiratory chain complexes. We examined the effects of ObgH1 and Mtg1 gene silencing on the formation of respiratory chain complexes. First, we analyzed the steady-state levels of the subunits of the respiratory chain complexes (Figure 4A). The ObgH1 knockdown caused no apparent change. On the other hand, on the Mtg1 knockdown, the levels of the subunits encoded by mitochondrial DNA moderately decreased, whereas the levels of the subunits encoded by genomic DNA were not altered. Next, we analyzed the assembly of respiratory chain complexes (Figure 4B). We performed Blue-Native PAGE followed by immunoblotting analyses. In mammals, the respiratory chains consist of five complexes (Complexes I, II, III, IV and V), and they contain giant complexes called supercomplexes, including Complexes I, III and IV (36). The Mtg1 knockdown predominantly reduced the formation of Complexes I and IV, and consequently the supercomplexes as well. The formation of Complex V was somewhat disordered as indicated by the appearance of the aberrant complex (Figure 4B,

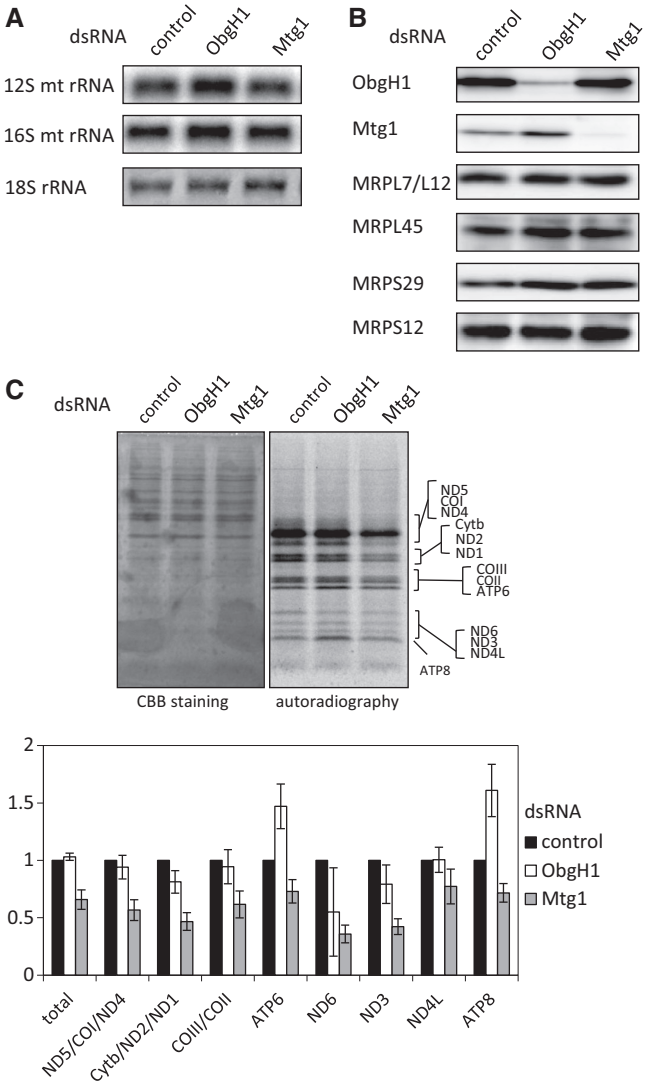

Figure 3. The effects of the knockdowns of ObgH1 and Mtg1 on mitochondrial ribosomes and translation. (**A**) Analysis of the steady-state levels of mitochondrial rRNAs. After HeLa cells were transfected with the indicated dsRNAs for 7 days, total RNA was extracted and analyzed by northern blotting, using probes specific for the 12S and 16S mitochondrial rRNAs. To confirm equal RNA loading, the cytoplasmic 18S rRNA was stained by ethidium bromide. (**B**) Analysis of the steady-state levels of mitochondrial ribosomal proteins. After HeLa cells were transfected with the indicated dsRNAs for 5 days, total cell lysates were analyzed by immunoblotting against the small subunit ribosomal proteins (MRPS29 and MRPS12) and the large subunit ribosomal proteins (MRPL7/L12 and MRPL45). (**C**) Analysis of mitochondrial translation activity. After HeLa cells were transfected with the indicated dsRNAs for 7 days, mitochondrial translation products were labeled with [^{35}S] methionine. Total cell lysates were separated by SDS–PAGE and visualized by autoradiography (upper right panel), and the intensities of the bands were quantified (lower). The value in the control dsRNA-treated cells was set to 1. The error bars represent standard deviations of three independent experiments. To confirm equal protein loading, the same gel was stained with CBB (upper left panel).

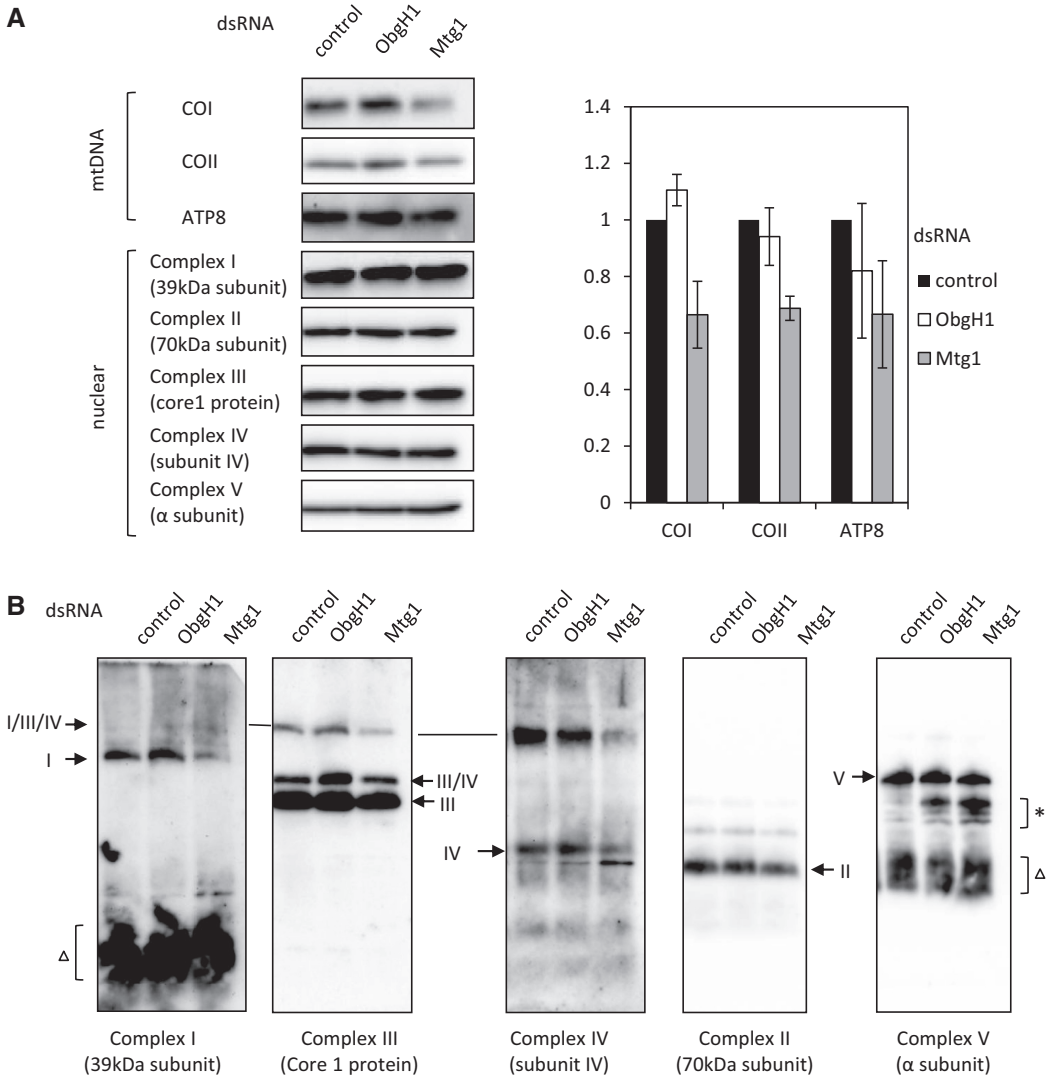

Figure 4. The effects of the knockdowns of ObgH1 and Mtg1 on the formation of respiratory chain complexes. (**A**) Analysis of the steady-state levels of the subunits of respiratory chain complexes. After HeLa cells were transfected with the indicated dsRNAs for 5 days, total cell lysates were subjected to immunoblot analyses. COI, COII and ATP8 are mtDNA-encoded subunits; the other subunits are encoded by the nuclear genome. The intensities of the bands were quantified for COI, COII and ATP8 (right). The value in the control dsRNA-treated cells was set to 1. The error bars represent standard deviations of three independent experiments. (**B**) Analysis of the assembly of respiratory chain complexes. After HeLa cells were transfected with the indicated dsRNAs for 5 days, mitochondrial fractions were separated by Blue-Native gel electrophoresis and subjected to immunoblot analyses. Arrows indicate the position of each complex. The complexes indicated with an asterisk represent the aberrant Complex V that reproducibly appears on ObgH1 or Mtg1 knockdown. Open triangles indicate unidentified non-specific bands.

asterisk). The changes in the formation of Complexes II and III were modest. On the other hand, the ObgH1 knockdown only moderately altered the assembly of Complex V, as indicated by the aberrant complex (Figure 4B, asterisk). This might correlate with the specific upregulation of the synthesis of the subunits of Complex V (ATP6 and ATP8) on the ObgH1 knockdown (Figure 3C). Considering the fact that all of the Complex II subunits are encoded by genomic DNA, it seems reasonable that neither the ObgH1 nor Mtg1 knockdown altered the formation of Complex II.

GTPase activities of ObgH1 and Mtg1

The Obg proteins have low intrinsic GTPase activity (22,37). On the other hand, the YlqF/YawG proteins have ribosome-dependent GTPase activity (23,26,27). We analyzed the GTPase activities of ObgH1 and Mtg1. The intrinsic GTPase activity of ObgH1 was observed, as previously reported (22). Mtg1 lacked intrinsic GTPase activity, but exhibited GTPase activity in 39S- and 55S-dependent manners (Figure 5A). The k_{cat} value of Mtg1 in the presence of the 55S mitochondrial ribosome was $0.031 \pm 0.001\,min^{-1}$ (Table 1 and Figure 5B). We did not detect the ribosome-dependent GTPase activity of ObgH1 (Supplementary Figure S2).

DISCUSSION

In the present study, we sought to elucidate the functions of two human G-proteins, ObgH1 and Mtg1, in the

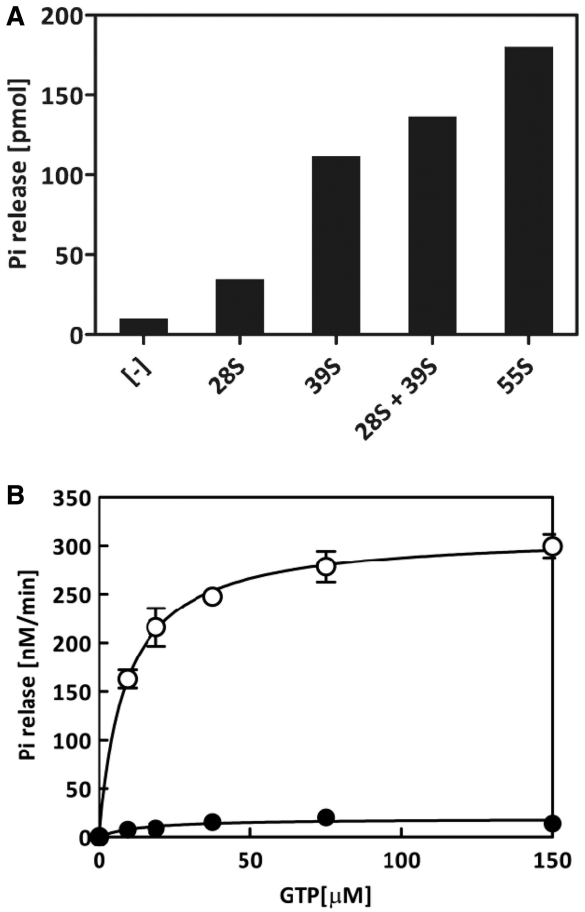

Figure 5. Ribosome-dependent GTPase activity of Mtg1. (**A**) Recombinant Mtg1 (f.c. 10 μM) was incubated with γ-[^{32}P]GTP in the presence of the indicated subunit of the mitochondrial ribosome (f.c. 0.2 μM), and the release of [^{32}P]Pi was measured. (**B**) A saturating amount of recombinant Mtg1 (f.c. 10 μM) was incubated with γ-[^{32}P]GTP in the presence (open circles) on absence (filled circles) of mitochondrial 55S ribosomes (f.c. 0.2 μM), and the release of [^{32}P]Pi was measured.

biogenesis of ribosomes in human mitochondria. ObgH1 and Mtg1 are localized in mitochondria, in association with the inner membrane and exposed on the matrix side (Figure 1), consistent with the fact that mitochondrial ribosomes interact with the inner membrane (3). Mtg1 and ObgH1 specifically associate with the large subunit of the mitochondrial ribosome, in GTP-dependent manner (Figure 2). The large ribosomal subunit stimulates the GTPase activity of Mtg1, whereas only the intrinsic GTPase activity was detectable with ObgH1 (Figure 5 and Table 1).

Our results suggested that Mtg1•GTP binds to the premature large ribosomal subunit and is released from the mature large ribosomal subunit after GTP hydrolysis, as proposed for the function of the bacterial homologue (27). It is likely that ObgH1•GTP also binds to the premature large ribosomal subunit. However, the means by which ObgH1 is released from the ribosome remain unclear. In *E. coli*, the dysfunction of ObgE leads to the accumulation of 50S subunit assembly intermediates, and the level of another assembly factor, RsfA/YbeB, is reduced in these assembly intermediates (21). It is possible that ObgH1 hydrolyzes GTP and is released from the premature large ribosomal subunit after mitochondrial RsfA (mtRsfA/C7orf30) is recruited to the ribosome.

Table 1. GTPase activities of ObgH1 and Mtg1

	k_{cat} (min^{-1})	Reference
ObgH1	0.014 ± 0.005	22
ObgH1 + 55S	ND	This study
Mtg1	ND	This study
Mtg1 + 55S	0.031 ± 0.001	This study

ObgH1 and Mtg1 were mixed with 0–150 μM GTP and incubated for 20 min at 30°C. The released phosphate was measured. Data represent mean ± SD of three independent experiments.
ND, not detected.

Mtg1 may actually be involved in mitochondrial ribosome assembly, and the knockdown of Mtg1 might lead to the decreased level of mature ribosomes. Consequently, on the Mtg1 knockdown, the overall mitochondrial translation activity was decreased (Figure 3C), and the defects in the formation of respiratory complexes were detected (Figure 4B). On the other hand, it is not still clear whether ObgH1 is indeed involved in the assembly of mitochondrial ribosomes. The depletion of ObgH1 led to the specific activation of the translation of the Complex V subunits (Figure 3C). The ObgH1 knockdown only moderately caused a change in the formation of Complex V (Figure 4B). Thus, ObgH1 is at least involved in mitochondrial translation and respiratory complex assembly. The translation of the Complex V subunits in mitochondria is affected by the assembly status of the respiratory complexes. For example, in yeast mitochondria, the translation of the Complex V subunits is contingent on the presence of F1 ATPase, a part of Complex V (38). The enhanced translation of the Complex V subunits accompanies the defects in the assembly of Complexes III and IV (39). The depletion of ObgH1 may cause an undetectable disorder in the assembly of respiratory complexes, and it might activate the translation of the Complex V subunits.

For the proper assembly of the respiratory complexes, the translating ribosomes must be in close proximity to the appropriate subunits of the respiratory complexes as well as to the assembly factors. ObgH1, as well as Mtg1, may play a role in the spatial coordination of the ribosome with either the respiratory complex or the assembly factors. It is noteworthy that NOA1/C4orf14, the human homologue of yeast Mtg3, might play this role. NOA1/C4orf14 interacts with Complex IV, and the knockdown of NOA1/C4orf14 causes defects in the assembly of Complex IV and the respiratory supercomplexes containing Complex IV (40).

In *E. coli* and *Caulobacter crescentus*, mutations in ObgE result in defects in chromosome replication and segregation, which occur before defects in ribosome assembly (28,41,42). Thus, ObgE has been suggested to couple DNA metabolism to the translational status (28). In

mammalian mitochondria, ObgH1 is one of the binding partners of ATPase family AAA domain-containing protein 3 (ATAD3), a mitochondrial nucleoid component involved in the maintenance and replication of mtDNA (43). It is plausible that ObgH1 may couple mtDNA metabolism and mitochondrial protein synthesis.

Future studies, such as the global identification of the binding partners of Mtg1 and ObgH1, are awaited to fully understand the functions of Mtg1 and ObgH1 in the biogenesis of mitochondrial ribosomes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–5.

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