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Mitochondrial tRNA cleavage by tRNA-targeting ribonuclease causes mitochondrial dysfunction observed in mitochondrial disease

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ABSTRACT

Mitochondrial DNA (mtDNA) is a genome possessed by mitochondria. Since reactive oxygen species (ROS) are generated during aerobic respiration in mitochondria, mtDNA is commonly exposed to the risk of DNA damage. Mitochondrial disease is caused by mitochondrial dysfunction, and mutations or deletions on mitochondrial tRNA (mt tRNA) genes are often observed in mtDNA of patients with the disease. Hence, the correlation between mt tRNA activity and mitochondrial dysfunction has been assessed. Then, cybrid cells, which are constructed by the fusion of an enucleated cell harboring altered mtDNA with a ρ^0 cell, have long been used for the analysis due to difficulty in mtDNA manipulation. Here, we propose a new method that involves mt tRNA cleavage by a bacterial tRNA-specific ribonuclease. The ribonuclease tagged with a mitochondrial-targeting sequence (MTS) was successfully translocated to the mitochondrial matrix. Additionally, mt tRNA cleavage, which resulted in the decrease of cytochrome *c* oxidase (COX) activity, was observed.

1. Introduction

Cells need energy to maintain their biological activity, and mitochondria are the cells' powerhouses. Mitochondria have genome that is distinct from nuclear DNA, and mitochondrial proteins are encoded by genes on both nuclear DNA and mitochondrial DNA (mtDNA). To date, 1013 genes encoding human mitochondrial proteins have been discovered [1], and more than 90% of these proteins are nuclear-encoded. On the other hand, human mtDNA encode only thirteen proteins, all of which are components of respiratory chain complexes [2]. Then mitochondria synthesize these proteins using mtDNA-encoded two rRNAs (12S and 16S rRNA) and twenty-two mitochondrial tRNAs (mt tRNA). These facts indicate that mtDNA integrity is important for maintenance of respiratory chain activity. During aerobic respiration, mitochondria carry out oxidative phosphorylation to generate ATP. This process also produces reactive oxygen species (ROS) that often induce DNA damage. Mitochondrial disease is caused by dysfunctional mitochondria, and mutations or deletions of mitochondria-associated nuclear genes and mtDNA genes can be predisposing factors of mitochondrial disorders. Among them, point mutations or deletions of mt tRNA genes, which occupy only 5–10% of mtDNA, are the major pathogenic factors [3], suggesting that the loss or decrease of mt tRNA activity leads to mitochondrial disease. For example, mitochondrial 3243 adenine-to-guanine mutation on mt $tRNA^{Leu(UUR)}$ gene is observed in patients with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) [4]. Similarly, patients suffering from myoclonic epilepsy with ragged-red fibers (MERRF) present the mitochondrial 8344 mutation, wherein an adenine residue within the TΨC loop of mt $tRNA^{Lys}$ is replaced by a guanine residue [5]. Some researchers have assessed *in vitro* respiratory chain deficiency by using cell lines like cybrid that harbor defective mtDNA to elucidate the correlation between mutations or deletions on mt tRNA genes and mitochondrial function [6–10]. These experiments demonstrated that aberrant mt tRNA gene is linked to mitochondrial dysfunction. However, it was also found that high accumulation of mutations or deletions on mt tRNA gene is required for the mitochondrial dysfunction [11], thus the underlying mechanism remains unclear. While cybrid cell lines are quite useful, their establishment is technically accomplished and time-consuming. Then others may try to directly introduce mutation or deletion into mt tRNA gene.

Abbreviations: mnm5U, 5-methylaminomethyluridine; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes; MERRF, myoclonic epilepsy with ragged-red fibers; MTS, mitochondrial-targeting sequence; TALEN, transcription activator-like effector nuclease; ZFN, zinc finger nuclease; COX, cytochrome *c* oxidase.

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Although genetic modification of mtDNA is difficult, a study involving mitochondria-targeting nucleases demonstrated reduction of mutant mtDNA [12]. Moreover, recent breakthroughs in genetic engineering, such as transcription activator-like effector nuclease (TALEN) and zinc finger nuclease (ZFN), have greatly contributed to advancement of genome modification technique, and resulted in elimination of pathogenic mtDNA [13,14]. While these technologies are promising, the flexibility to target sequences is limited and needs to be improved.

Here, we present a different approach of inducing mt tRNA dysfunction with tRNA-targeting ribonuclease, colicin D. Colicin D is a bacterial toxin encoded by a plasmid, and impairs translation of other sensitive *Escherichia coli* cells that do not possess the same plasmid. We have previously reported that colicin D is a $tRNA^{Arg}$-specific ribonuclease in *E. coli* cells [15]. Therefore, we hypothesized that mt tRNA cleavage by colicin D could impair mt tRNA activity, which mimics mitochondrial dysfunction observed in mitochondrial disease. Then mitochondrial-targeting sequence (MTS) was fused with the active domain of colicin D (D-CRD), and the recombinant protein was expressed in HeLa cells. The tagged ribonuclease was translocated to the mitochondrial matrix, and it specifically cleaved mt tRNA. This resulted in a decrease in respiratory chain activity.

2. Materials and methods

2.1. Cell culture

HeLa cells were grown in DMEM (high glucose) supplemented with 10% FBS at 37 °C in an atmosphere of 5% CO_2.

2.2. Plasmid construction

The gene encoding colicin D 595–697 amino acid sequence, which includes D-CRD and the stop codon, was PCR-amplified to generate *Xho*I and *Xba*I sites at the 5′ and 3′ termini, respectively. Similarly, *Not*I and *Xho*I sites were fused to the 5′ and 3′ ends of a green fluorescent protein (GFP)-coding gene, respectively. The MTS-coding region derived from human cytochrome *c* oxidase subunit VIII (COX8) was amplified so that it contains a *Hin*dIII site, the kozak sequence, the start codon at the 5′ end, and a *Not*I site at the 3′ end. These three DNA fragments were inserted in the corresponding restriction sites of pcDNA3.1 (+) (Life Technologies, Carlsbad, CA, USA). The resulting cells carrying the plasmid expressed a fusion protein consisting of the GFP-tagged MTS and D-CRD (COX8-GFP-D). Additionally, gene encoding D-CRD (H611Y), which is catalytically inactive, was cloned instead of the wild-type gene, and used as a control (COX8-GFP-D (H611Y)). To prepare a plasmid expressing the fusion protein lacking the MTS (GFP-D), the plasmid described above (pcDNA-*COX8-GFP-D*) was digested with *Hin*dIII and *Xho*I, and replaced with PCR-amplified genes for kozak sequence, the start codon, and *gfp*. Gene encoding a specific inhibitor of colicin D (ImmD) was amplified to introduce a FLAG-tag coding region at the 5′ end, and cloned into the *Pst*I–*Bam*HI sites of pCAX2 [16].

2.3. Cloning of cleaved half tRNA

The preparation of purified D-CRD was described previously [17]. Sixteen micrograms of mitochondrial total RNA was pre-incubated in 20 μL of buffer containing 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 100 μg/mL BSA at 70 °C for 5 min to refold the tRNA structure. Ninety picomoles of purified D-CRD were then added and the incubation was continued at 37 °C for 15 min. To detect cytosolic tRNA susceptible to D-CRD, 50 μg of total tRNA and 125 pmol of D-CRD were used instead. The RNA solution was mixed with the same volume of loading solution (9 M urea, 0.02% BPB, and 0.02% XC), and electrophoresed using a 10% polyacrylamide gel containing 7 M urea, 89 mM Tris–borate (pH 8.3), and 2 mM EDTA. tRNA fragments were visualized with SYBR Green II RNA Gel Stain (Life Technologies), excised, and then eluted. RNA fragment cloning was performed with Small RNA Cloning Kit (TaKaRa, Tokyo, Japan).

2.4. Localization analysis by fluorescent microscopy

Two micrograms of pcDNA-*COX8-GFP-D*, pcDNA-*COX8-GFP-D* (*H611Y*), or pcDNA-*GFP-D* (*H611Y*) were combined with 2 μg of pCAX-*ImmD* and used to transfect HeLa cells in 6-well plates (80–90% confluence) with Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. The next day, the cells were appropriately diluted and seeded into 24-well plates containing cover glasses, and cultured for 1 d. Cells were then treated with fresh medium containing 100 μM MitoTracker Red CMX-Ros (Life Technologies) at 37 °C for 15 min and fixed with 3.7% formaldehyde. Cells were permeabilized with cold methanol, and then were treated with 10% FBS in PBS (−) to avoid non-specific interaction of the antibodies. ImmD was detected using an ImmD-specific polyclonal antibody and Alexa Fluor 350 goat anti-rabbit IgG (H+L) (Life Technologies).

2.5. Cell fractionation and Western blotting

HeLa cells plated in 100-mm dishes (80–90% confluence) were transfected with 6 μg of pcDNA-*COX8-GFP-D* (*H611Y*) and 6 μg of pCAX-*ImmD* mixed with 30 μL of Lipofectamine 2000. The next day, cells were transferred two 100-mm dishes and cultured for 1 d. Cells were then washed with cold PBS (−), harvested, resuspended in 2 mL of buffer containing 10 mM Tris–HCl (pH 7.5), 10 mM NaCl, and 1.5 mM $MgCl_2$, and kept on ice for 10 min. Cells were then homogenized, and 2 mL of 12.5 mM Tris–HCl (pH 7.5), 525 mM mannitol, 175 mM sucrose, and 2.5 mM EDTA was added. Cells were then centrifuged at 1300*g* for 5 min at 4 °C to exclude nuclei. The supernatant was centrifuged at 17,000*g* for 15 min at 4 °C, and the resultant supernatant was collected and used as the cytosolic fraction. The pellet, containing crude mitochondria, was washed twice with buffer containing 5 mM Tris–HCl (pH 7.5), 210 mM mannitol, 70 mM sucrose, and 1 mM EDTA. Protein concentration was determined using the Bradford method. Ten micrograms of protein were loaded onto a 10% gel to detect COX8-GFP-D (H611Y), while a 14% gel was used to detect succinate dehydrogenase complex subunit B (SDHB), β-actin, and ImmD. To detect COX8-GFP-D (H611Y), a D-CRD specific polyclonal antibody or an anti-GFP (MBL, Nagoya, Japan) and a goat HRP-conjugated anti-rabbit IgG (Upstate Biotechnology, Lake Placid, NY, USA) were used as primary and secondary antibodies, respectively. SDHB was detected using a rabbit polyclonal IgG SDHB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a goat HRP-conjugated anti-rabbit IgG, (Upstate Biotechnology). ImmD was detected using the ImmD-specific polyclonal antibody.

2.6. Northern hybridization

HeLa cells at 80–90% confluence in 6-well plates were transfected with 2 μg of pcDNA-*COX8-GFP-D*, pcDNA-*COX8-GFP-D* (*H611Y*), or pcDNA-*GFP-D* combined with 2 μg of pCAX-*ImmD*. The next day, the cells were passaged in 60-mm dishes and cultured for another day. Mitochondria isolation was performed as described above. Cytosolic and mitochondrial total RNA was prepared with ISOGEN (Nippongene, Tokyo, Japan). Northern hybridization was performed as described previously [15], and 2 μg of total RNA was

loaded. The primer sequences were as follows: mt tRNA-His: 5′-GTCGTAAGCCTCTGTTGTCA-3′; mt tRNA-Asp: 5′-TTGGCAAAGTTATGAAATGGTTTTTCTAATACCTT-3′; 5S rRNA: 5′-TTCCGAGATCAGACGAGATC-3′. cyto tRNA-Lys: 5′-TTGAACCCTGGACCCTCAGA-3′.

The oligonucleotide 3′ ends were DIG-labeled with DIG Oligonucleotide 3′-End Labeling Kit, 2nd Generation (Roche, Basel, Switzerland). After hybridization, the membrane was washed, treated with blocking solution, and the signal was detected with anti-digoxigenin-AP Fab fragment (Roche) and CDP-Star (Roche).

2.7. COX activity assay

HeLa cells plated in 60-mm dishes (80–90% confluent) were transfected with 4 μg of pcDNA-*COX8-GFP-D* or pcDNA-*COX8-GFP-D* (*H611Y*) combined with 4 μg of pCAX-*ImmD*. Transfection of pcDNA-*COX8-GFP-D* (*H611Y*) was duplicated for the addition of chloramphenicol. The next day, cells were trypsinized and plated onto 60-mm and 10-mm dishes and cultured. At this time, chloramphenicol was added to the dish expressing COX8-GFP-D (H611Y) at a final concentration of 500 μg/mL. Two days after transfection, the cells in 100-mm dish were harvested, and mitochondria were isolated as described above. Total RNA was prepared from the cells plated in 60-mm dishes and used for Northern blotting. Isolated mitochondria were resuspended in enzyme dilution buffer containing 10 mM Tris–HCl (pH 7.0), 250 mM sucrose, and 1 μM *n*-dodecyl-β-D-maltoside. The mitochondrial concentration was determined using the Bradford method. COX activity was measured using the Cytochrome *c* Oxidase Assay Kit (SIGMA–Aldrich, Saint Louis, MO, USA); 1 μg of mitochondria was applied to the assay. All procedures were performed according to the manufacturer's protocol.

3. Results

3.1. mt $tRNA^{His}$ is susceptible to D-CRD

We previously showed that colicin D specifically cleaves four *E. coli* $tRNA^{Arg}$s, $tRNA^{Arg}_{ICG}$, $tRNA^{Arg}_{CCG}$, $tRNA^{Arg}_{mnm5UCU}$, and $tRNA^{Arg}_{CCU}$ [15]. The cleavage site is between positions 38 and 39, at the 3′ end of the anticodon-loops, and 2′, 3′ cyclic phosphate and 5′ OH termini are yielded [15]. Additionally, among *E. coli* $tRNA^{Arg}$s, $tRNA^{Arg}_{ICG}$ is the most susceptible to colicin D, followed by $tRNA^{Arg}_{CCG}$. Our biochemical studies indicated that a guanine residue as the third base of the anticodon enhances the susceptibility to colicin D, which explains the high susceptibility of $tRNA^{Arg}_{ICG}$ and $tRNA^{Arg}_{CCG}$. Human mtDNA encodes five tRNAs containing guanine as the third base of the anticodon, mt $tRNA^{Arg}$, mt $tRNA^{Gln}$, mt $tRNA^{His}$, mt $tRNA^{Leu(CUN)}$, and mt $tRNA^{Pro}$. Our results suggest that all of them are susceptible to colicin D. Moreover, tRNAs containing uracil as the third base also seem susceptible; $tRNA^{Arg}_{mnm5UCU,}$ and $tRNA^{Arg}_{CCU}$ are actually susceptible to colicin D, but to a lesser extent than $tRNA^{Arg}_{ICG}$ and $tRNA^{Arg}_{CCG}$ in *E. coli* cells. Thus, to determine which mt tRNA species were cleavable by D-CRD, total mt tRNA was incubated with purified D-CRD. Released tRNA halves were reverse-transcribed, cloned, and sequenced. Fourteen clones out of twenty-four clones, including three clones derived from cytosolic tRNAs, sequenced were mt $tRNA^{His}$ halves (Supplementary Table I). Therefore, mt $tRNA^{His}$ was used to evaluate the cleavage activity of D-CRD in mitochondria.

3.2. COX8-GFP-D targets mitochondria

A previous report showed that GFP with COX8 at the N-terminal (COX8-GFP) localized in the mitochondrial matrix [18]. Thus, D-CRD starting at the 595th residue [17] was fused at the C-terminal of COX8-GFP (COX8-GFP-D) and transiently expressed in HeLa cells (Fig. 1A). Generally, nuclear-encoded mitochondrial proteins are translated on free ribosomes, suggesting that COX8-GFP-D cleaves cytosolic tRNA in the process of targeting mitochondria. Thus, ImmD, which interacts with D-CRD to inhibit tRNA cleavage activity, was also expressed in the cytosol to avoid the cleavage of cytosolic tRNA. Mitochondrial precursor proteins are unfolded once they reach the mitochondrial surface, by which ImmD is expected to be separated from D-CRD. COX8-GFP-D (H611Y), including a catalytically inactivated D-CRD [15], or GFP-D were also expressed with ImmD as controls. To verify that COX8-GFP-D localized in mitochondria, fluorescent microscopic analysis was first carried out. Cells were fixed, and COX8-GFP-D, COX8-GFP-D (H611Y), GFP-D (H611Y), and ImmD were detected by immunofluorescence (Fig. 1B). Our results showed that COX8-GFP-D (H611Y) immunofluorescence merged with that of mitochondria, which were visualized by staining with MitoTracker, while ImmD was uniformly expressed in the cytosol. Additionally, GFP-D (H611Y) presented the same pattern as ImmD, indicating that COX8-GFP-D (H611Y) localizes in the mitochondria in a MTS-dependent manner. Wild-type COX8-GFP-D also overlapped with that of mitochondria, however, some fluorescence was also detected in the cytosol. Then, to assess the localization in more detail, COX8-GFP-D (H611Y) and ImmD were co-expressed, and cells were fractionated. The fractions were used for Western blot analysis (Fig. 1C). ImmD and SDHB, which are used as markers of mitochondrial fraction, were detected in the cytosol and mitochondria fraction, respectively, showing that the mitochondria were properly isolated, and ImmD remained in the cytosol. Two bands were detected in the cytosol fraction with the D-CRD-specific antibody, and the upper band disappeared in the mitochondrial fraction, indicating that the upper and lower bands corresponded to the precursor and the mature form, respectively. In the mature form, the COX-tag was cleaved during the translocation process. Unexpectedly, three extra bands were detected with the anti-GFP antibody. The major band migrated above the 31-kDa marker. These bands were not observed when the D-CRD-specific antibody was used instead of the anti-GFP antibody, indicating that the C-terminal D-CRD region of COX8-GFP-D (H611Y) was unstable in mitochondria.

3.3. COX8-GFP-D was translocated to the mitochondrial matrix and specifically cleaves mt $tRNA^{His}$

To verify that COX8-GFP-D localizes in the mitochondrial matrix and cleaves mt tRNA, total tRNA was prepared from COX8-GFP-D-expressing cells and subjected to northern hybridization analysis (Fig. 2A). The band intensity of intact mt $tRNA^{His}$ decreased when the wild-type COX8-GFP-D was expressed, while this was not observed in cells expressing COX8-GFP-D (H611Y) or GFP-D. Additionally, mt $tRNA^{Asp}$, which was suggested to be resistant to the cleavage according to the RNA cloning described above, was not decreased in cells expressing the wild-type COX8-GFP-D. Taken together, our results indicate that COX8-GFP-D localizes in the mitochondrial matrix and cleaves mt $tRNA^{His}$.

In our experiments, ImmD was co-expressed with COX8-GFP-D to inhibit cytosolic tRNA cleavage. Thus, cytosolic tRNA status was also examined to assess whether ImmD appropriately protected cytosolic tRNA from cleavage. Prior to the experiment, cytosolic tRNAs susceptible to D-CRD were determined by tRNA fragment cloning and sequencing as described above. The 3′ half of $tRNA^{Lys}_{UUU}$ was the most cloned among the cytosolic tRNAs (Supplementary Table II). Thus, $tRNA^{Lys}_{UUU}$ status was used to evaluate cytosolic tRNA cleavage. Plasmids were transfected with the same combination presented in Fig. 2A, except that pcDNA-*GFP-D* was mixed with the empty pCAX2 vector instead of pCAX-*ImmD*. Total RNA was then

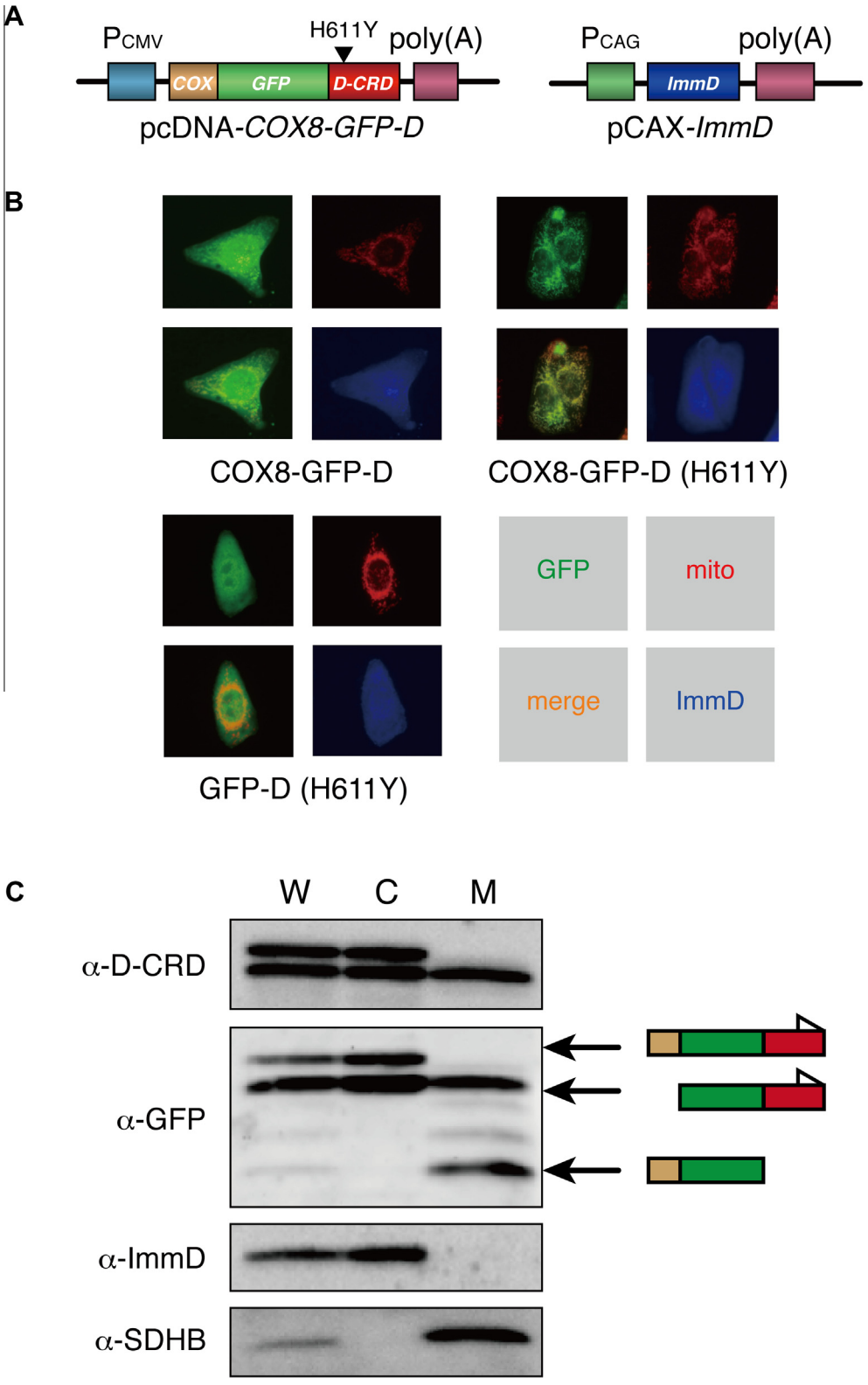

Fig. 1. COX8-GFP-D localizes in mitochondria. (A) Schematic representation of plasmids expressing COX8-GFP-D and ImmD. COX8-GFP-D (H611Y) includes an amino acid substitution of His611 to Tyr within D-CRD, indicated by a solid arrowhead. (B) Localization of COX8-GFP-D, COX8-GFP-D (H611Y), GFP-D (H611Y), and ImmD detected by immunofluorescence microscopy. Fluorescence derived from GFP was used to determine the localization of COX8-GFP-D, COX8-GFP-D (H611Y), and GFP-D (H611Y). Mitochondria were stained with MitoTracker and the signal was merged with that of GFP. ImmD was stained with specific antibody and fluorescent dye-labeled antibody. (C) Detection of COX8-GFP-D (H611Y) and ImmD from fractionated cell lysate by Western blotting. W, C, and M labels at the top of panels indicate whole cell, cytosol, and mitochondria lysates, respectively. The primary antibodies used are indicated on the left of panels, and possible form of COX8-GFP-D (H611Y) is drawn on the right.

extracted and used for northern hybridization analysis with a cytosolic $tRNA^{Lys}_{UUU}$-specific probe (Fig. 2B). Cytosolic $tRNA^{Lys}_{UUU}$ was not cleaved in cells expressing both COX8-GFP-D and ImmD. The fragment of cytosolic $tRNA^{Lys}_{UUU}$ was actually detected when GFP-D alone was expressed, indicating that COX8-GFP-D expressed with ImmD does not cleave cytosolic tRNAs in this system.

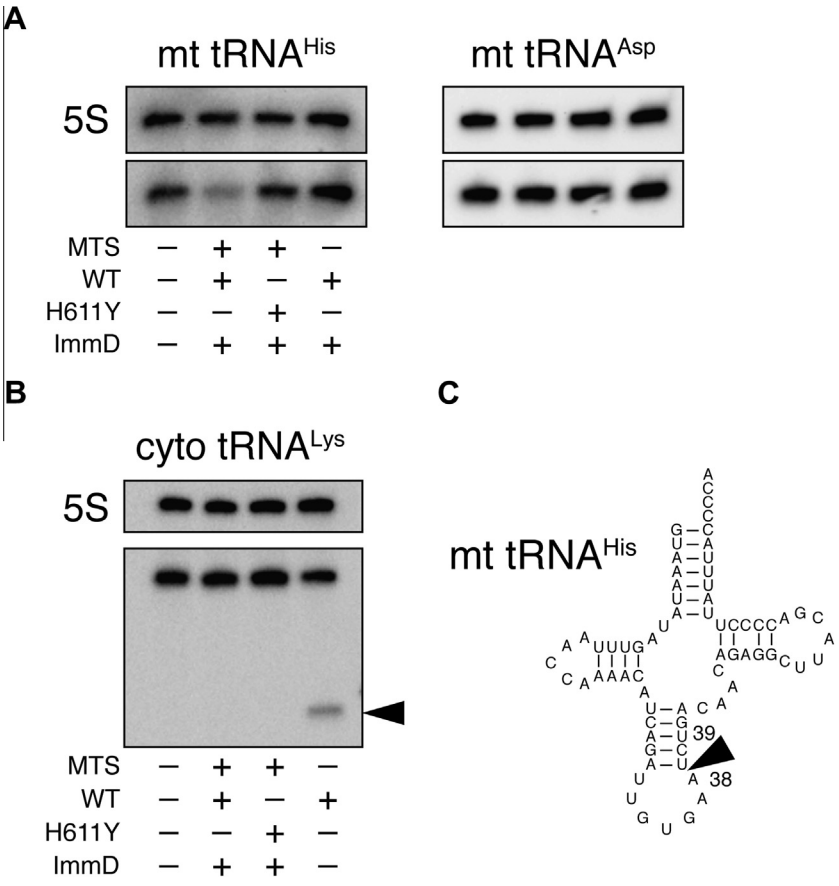

Fig. 2. COX8-GFP-D specifically cleaves mt $tRNA^{His}$ in the mitochondrial matrix. (A) Total tRNA was prepared from cells expressing COX8-GFP-D, COX8-GFP-D (H611Y), or GFP-D together with ImmD, and mt $tRNA^{His}$ and mt $tRNA^{Asp}$ were then detected by Northern hybridization analysis. In the left most lane, total RNA was prepared from cells transfected with two empty vectors (pcDNA3.1 (+) and pCAX2). 5S rRNA was detected as a loading control. (B) Total tRNA was prepared from cells expressing COX8-GFP-D or COX8-GFP-D (H611Y) with ImmD, and cytosolic $tRNA^{Lys}_{UUU}$ cleavage was analyzed by northern hybridization. On the most right lane, total RNA from cells expressing GFP-D alone was loaded. tRNA half released from cleaved $tRNA^{Lys}_{UUU}$ is indicated by a solid arrowhead. (C) Cleavage site of mt $tRNA^{His}$ judged by sequence determination of cleaved tRNA half. The numbers 38 and 39 indicate nucleotide positions adjacent of the cleavage site.

3.4. mt tRNA cleavage by COX8-GFP-D decreases COX activity

Human mtDNA encodes 13 proteins and all of them are components of the electron transport chain, indicating that mitochondrial translation impairment influences on the formation of respiratory chain complexes, which results in reduction of the electron transport chain activity. COX, which catalyzes the final step of the mitochondrial electron transport chain reaction, is composed of 13 subunits, and three of them are encoded by mtDNA. Therefore, the activity of cytochrome *c* oxidase (COX activity) was measured to evaluate the effect of mt tRNA cleavage (Fig. 3). Mitochondria were isolated from cells transfected as indicated, and COX activity was determined. Simultaneously, mt $tRNA^{His}$ cleavage was analyzed by northern hybridization analysis. Expression of COX8-GFP-D (H611Y) did not lead to the cleavage of mt $tRNA^{His}$ as observed in Fig. 2A, and COX activity remained intact. On the other hand, treatment of chloramphenicol, which impairs mitochondrial protein synthesis, reduced COX activity. When the wild-type COX8-GFP-D was expressed, mt $tRNA^{His}$ cleavage was observed and COX activity decreased. Together, these results indicate that COX8-GFP-D translocated to the mitochondrial matrix and cleaved mt $tRNA^{His}$, which resulted in a decrease of the electron transport chain activity.

4. Discussion

In this study, COX8-GFP-D was successfully translocated to the mitochondrial matrix in a MTS-dependent manner, and it cleaved mt tRNA. The sequencing analysis of the cleaved tRNA halves showed that both mitochondrial and cytoplasmic tRNAs possessed guanine or uracil as the third base of the anticodon (Supplementary Tables I and II). These results are in agreement with previous data indicating that *E. coli* tRNAs susceptible to D-CRD contain guanine or uracil at the same position. Although D-CRD prefers guanine to uracil at the third letter, the half of mt $tRNA^{His}$, which contains uracil at the position, was the most replicated fragment among total mt tRNA. This might reflect the level of each tRNA among total tRNA in the cells, and this was also observed for the cytosolic $tRNA^{Lys}_{UUU}$. When mt $tRNA^{His}$ was cleaved in the mitochondrial matrix, the cleaved fragment was not detected by northern hybridization (Figs. 2A and 3), showing that the mt tRNA half is not stable in the mitochondrial matrix. Fluorescent microscopic analysis suggested that not all of wild-type COX8-GFP-D localized in the mitochondria, some of which remained in the cytosol (Fig. 1B). Considering the fact that COX8-GFP-D (H611Y) fluorescent signal completely overlapped with that of mitochondria, the difference in localization between wild-type COX8-GFP-D and COX8-GFP-D (H611Y) may depend on the tRNA cleavage activity. The membrane potential ($\Delta\Psi$) is the driving force for targeting of the mitochondrial matrix by proteins. mt tRNA cleavage might induce a decrease in $\Delta\Psi$, which impaired the translocation of COX8-GFP-D itself. tRNA cleavage was not observed in the cytosol of cells expressing COX8-GFP-D and ImmD (Fig. 2B); hence, we believe that the partial translocation impairment, if any, did not cause cytosolic tRNA cleavage by COX8-GFP-D.

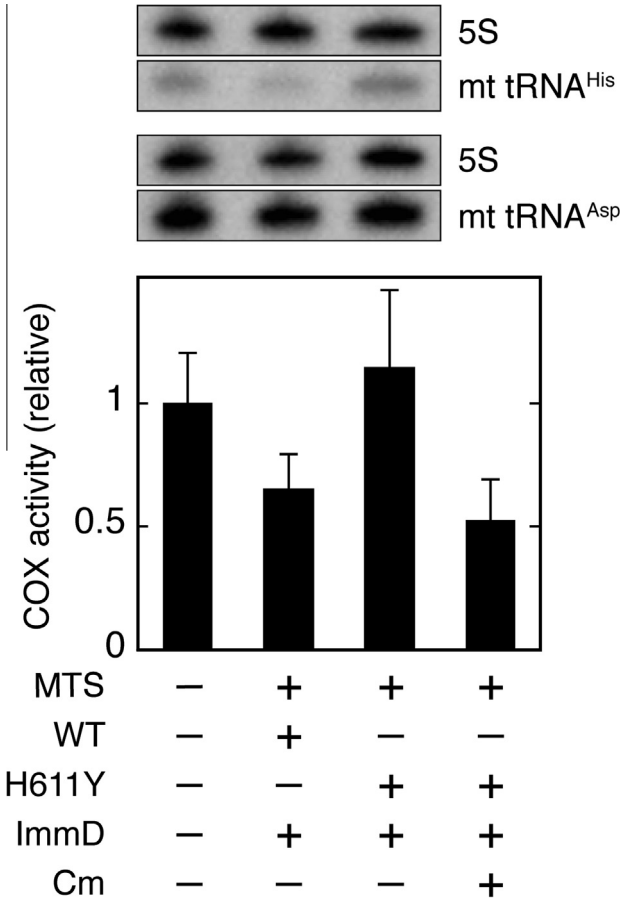

Fig. 3. COX8-GFP-D decreases the electron transport chain activity via mt tRNA cleavage. COX8-GFP-D or COX8-GFP-D (H611Y) was expressed with ImmD in HeLa cells, and cytochrome *c* oxidase activity of isolated mitochondria was measured. mt $tRNA^{His}$ cleavage was also detected and is presented above the histogram. 5S rRNA was detected as a loading control as shown in Fig. 2A and B. In the most right lane, cells expressing COX8-GFP-D (H611Y) and ImmD were treated with 500 μg/mL chloramphenicol, and the cytochrome *c* oxidase activity was assayed. Error bar indicates standard error calculated from three experiments.

Localization of COX8-GFP-D was also confirmed by cell fractionation and western blotting analysis. MTS cleavage was observed as reported previously. Additionally, higher mobility bands were detected when the anti-GFP antibody was used, which was not observed when the D-CRD-specific antibody was used (Fig. 1C). This indicates that D-CRD was degraded, while GFP was stable in the mitochondrial matrix. In spite of the instability of D-CRD in the mitochondrial matrix, COX-8-GFP-D successfully cleaved mt tRNA and reduced COX activity (Fig. 3). Although ImmD expressed in the cytosol presented a FLAG tag at the N-terminal, it was not detected with the anti-FLAG antibody (data not shown), indicating that the tag was also unstable in the cytosol.

This study describes an easy and reproducible method to investigate mt tRNA dysfunction without using cybrid cells or modification of mtDNA. Moreover, it will be useful to analyze cellular responses at an early stage upon the emergence of mt tRNA gene mutations or deletions. Mitochondria fuse and divide to functionally complement each other, and mitochondria with aberrant mt tRNAs interact with other mitochondria to receive intact mt tRNAs [19,20]. Thus, emergence of aberrant mt tRNA may not immediately induce mitochondrial dysfunction, rather the breakdown of the mitochondrial complementation might lead to mitochondrial disease. In cybrid cells, the analysis may be limited by the steady-state of cellular activity, due to the amount of time necessary for establishment of these cell lines. We provide a new approach and new insights on the mechanism underlying mitochondrial disease caused by mt tRNA dysfunction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.084.

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