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Association of a Novel Mitochondrial Protein M19 with Mitochondrial Nucleoids

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We have identified a novel mitochondrial protein, termed M19, by proteomic analysis of mitochondrial membrane proteins from HeLa cells. M19 is highly conserved among vertebrates, and possesses no homologous domains with other known proteins. By northern and western blotting, mouse M19 was shown to be expressed in various tissues, and to be especially abundant in the brain. Human M19 (hM19) is present in mitochondria, and protease-protection experiment showed it to be sublocalized in the matrix space. Carboxy-terminally tagged hM19 appeared as spotted signals within mitochondria and co-localized with signals arising from mitochondrial DNA (mtDNA), suggesting the inclusion of M19 in the mtDNA–protein complex (mitochondrial nucleoids). Fractionation of mitochondrial nucleoids from HeLa cells revealed that hM19 has a similar distribution pattern like that of known nucleoid components, such as mtSSB and PHBs, and surely exists in the nucleoid fraction. Furthermore, expression of M19 is closely related to the amount of mtDNA, because it was down-regulated in mtDNA-depleted ρ^0 HeLa cells. These results indicate that M19 associates with the nucleoid and likely regulates the organization and metabolism of mtDNA.

Key words: mitochondria, mitochondrial protein, mitochondrial nucleoids, mtDNA.

Abbreviations: CPS1, carbamoyl-phosphate synthetase 1; cyt.c, cytochrome c; FISH, Fluorescent *in situ* hybridization; LONP1, lon peptidase 1; mtDNA, mitochondrial DNA; mtSSB, mitochondrial single-stranded DNA-binding protein; OPA1, optic atrophy 1; PDHE2, pyruvate dehydrogenase E2; PDIP38, polymerase delta interacting protein 38; PHB, prohibitin; PHBs, prohibitin proteins; PMF, post-mitochondrial faction; TFAM, mitochondrial trnscription factor A; WCE, whole cell extract.

Mitochondria, which are membrane-enclosed organelles, are sometimes called the 'cellular power plant' because they generate most of the cellular ATP through oxidative phosphorylation. It is estimated that mammalian mitochondria consist of approximately 1,500 proteins, and about half of these proteins are known to be localized in and functionally associated with mitochondria (*1*). It has also been demonstrated that the composition of mitochondrial proteins varies between tissues and with age (*2*, *3*). Significant portions of mitochondrial proteins are membrane-associated, making them difficult to solubilize. Therefore, although mitochondrial proteome analysis has been extensively performed by many groups, complete cataloging of mitochondrial proteins has not been achieved.

Mitochondria are semi-autonomous organelles that contain their own genomic DNA, called mitochondrial DNA (mtDNA). It has been shown that mtDNA exists not as a naked structure, but rather as a highly organized structure associated with many proteins (the nucleoprotein complex); these complex structures are called mitochondrial nucleoids (*4*). The nucleoid components are believed to regulate stability, replication, transcription and segregation of mtDNA (*5*). Proteins associated with mtDNA have been well characterized in yeast; however, those in higher eukaryotes remain poorly understood. The composition of mitochondrial nucleoids appears to be different between yeast and higher eukaryotes: judging from the lack of homology in the primary amino acid structures, many of the yeast nucleoid proteins have no corresponding proteins in higher eukaryotes. There are at least two common proteins associated with mtDNA found in organisms ranging from bacteria to humans: mitochondrial transcription factor A (TFAM) and mitochondrial single-stranded DNA-binding protein (mtSSB). TFAM is known to be a major nucleoid protein, and works as a transcription factor for mtDNA and also as a factor that maintains the copy number of mtDNA (*6–8*). mtSSB, a functional and structural relative of bacterial SSB, is known to be associated with mtDNA in all eukaryotes. mtSSB is involved in the replication of mtDNA by stimulating its unwinding, stabilizing the single-strand regions and enhancing the rate of DNA polymerase activity (*9*, *10*). Nucleoid components in higher organisms have only recently begun to be identified (*11–13*).

In this study, we identified a novel mitochondrial protein, termed M19, by proteome analysis of

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mitochondria from HeLa cells. M19 is a highly conserved protein among vertebrates that contains no homologous domains with other proteins. By northern and western blot analyses, mouse M19 was shown to be expressed in various mouse tissues, and to be especially abundant in the brain. Human M19 (hM19) is sublocalized in the mitochondrial matrix space, and C-terminally FLAG-tagged M19 is localized as spotted signals within mitochondria, as is mtDNA. These observations suggest that M19 is included in the mitochondrial nucleoids. By immunoprecipitation and mass analysis, it was found that hM19 potentially interacts with recently identified mitochondrial nucleoid components, such as mtSSB, carbamoyl-phosphate synthase I (CPS1), lon peptidase 1 (LONP1) and polymerase delta interacting protein 2 (POLDIP2, also known as PDIP38) (*12*, *13*). Fractionation of mitochondrial nucleoids from HeLa cells further revealed that hM19 has a distribution pattern similar to that of known nucleoid components, such as mtSSB and PHB1/PHB2, and surely exists in the heavy fraction containing mtDNA and TFAM. Furthermore, expression of M19 is closely related to the amount of mtDNA, because it is decreased in ρ^0 HeLa cells that are cell lines completely lacking mtDNA. These results demonstrate that a novel mitochondrial protein M19 associates with the nucleoids and is a potential regulator of the organization and metabolism of mtDNA.

MATERIALS AND METHODS

Plasmid Construction—The coding region of hM19 was amplified by PCR from HeLa cell cDNA. The PCR product with 3× FLAG tag sequences or GFP-coding sequences at the 3′ termini was introduced into mammalian expression vector pCMV-SPORT (Invitrogen) as described previously (*14*). The coding region of hM19 was also inserted into the pGEX-4T-3 (Amersham Biosciences), pQE32 (Qiagen) or pDsRed1-N1 (Clontech) vectors. For RNA interference (RNAi), a small interfering RNA (siRNA) sequence for M19 (5′-GAGTACAAGCTGAT CCTGT-3′) was introduced into the pSilencer 3.1-H1 Puro vector (Ambion).

Cell Culture and Transfection—HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in an atmosphere containing 5% CO_2. ρ^0 HeLa cells were cultured in RPMI1640 containing 10% fetal bovine serum, pyruvate (0.11 mg/ml), uridine (0.05 mg/ml) and gentamicin (0.05 mg/ml). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Mitochondria in living HeLa cells were stained with MitoTracker Red CM-H_2XRos (250 nM, Molecular Probes) for 30 min at 37°C or with PicoGreen solution (3 μl/ml, Molecular Probes) for 60 min at 37°C. Cells expressing pSilencer 3.1-H1 Puro constructs were selected with 2 μg/ml of puromycin (Sigma).

Cell Fractionation and Preparation of Mitochondrial Nucleoids—Preparation of nuclear extract was followed by the method (*15*). HeLa cells were suspended in 0.25 M sucrose supplemented with 0.2 mM EDTA (sucrose solution) and broken by 10 strokes of a tight-fitting homogenizer. The suspension was centrifuged at 240*g* for 10 min to separate nuclear pellets and cytoplasmic fraction. The cytoplasmic fraction was further centrifuged at 10,000*g* for 10 min to separate mitochondrial pellets and post-mitochondrial fractions (PMFs). Mitochondrial nucleoids were prepared using velocity sedimentation (*13*). Briefly, isolated mitochondria were suspended in about one hundred volumes of digitonin solution (0.5 mg/ml digitonin in sucrose solution), and incubated at 4°C for 20 min with periodic mixing. After centrifugation at 10,000*g* for 10 min, the pellets were washed twice with sucrose solution, and collected as mitoplasts. About 10 volumes of detergent solution (1.2% Triton-X 100, 30 mM HEPES, pH 8, 2 mM EDTA, 2 mM DTT and 70 mM NaCl) were added to the mitoplasts, and mixed on ice for 5 min. After centrifugation at 3,000*g* for 5 min, the supernatant was loaded on top of a step-wise glycerol gradient as described in (*13*), and centrifuged at 186,000*g* in an SW41 rotor (Beckman) for 1.5 h. After centrifugation, fractions (1 ml each) were collected from the top.

Immunocytochemistry—HeLa cells were plated on 35-mm poly-L-lysine-coated glass-bottomed dishes (Matsunami Glass Ind.) and fixed for 20 min at room temperature with 4% paraformaldehyde and 0.4% Triton X-100 in PBS. The cells were probed with antibodies against FLAG (Sigma, rabbit polyclonal antibody) and cytochrome *c* (BD Pharmingen, mouse monoclonal antibody) as previously described (*14*). Fluorescent images were captured and analysed with a μRadiance™ Laser Scanning Confocal Microscope System (Bio-Rad).

Combined Immunofluorescence and Fluorescent In Situ Hybridization—Cells were fixed for 15 min with 4% formaldehyde in PBS. The cells were then treated for 15 min with 0.1% Triton X-100 in PBS, and probed with anti-M19 rabbit polyclonal antibody (described bellow). After labeling with Cy3-conjugated anti-rabbit IgG (Molecular Probes), the cells were fixed for 20min with 4% paraformaldehyde in PBS and treated for 15 min with 0.1% Triton X-100 in PBS. Fluorescent *in situ* hybridization (FISH) was basically followed by the method (*16*). Two μg of 1.1 kb D-loop mtDNA fragment (16,029–599 nt) was used in a nick translation reaction (Roche) to label it with Alexa Fluor 488 fluorescent dye (ARES DNA labeling kit, Molecular probes). Fluorescent images were captured with Axio Observer D1 system (Carl Zeiss).

Protease-protection Assay and Membrane Association Assay—For the protease-protection assay, mitochondria were treated for 20 min at room temperature with 0.25 mg/ml trypsin (Sigma) in a sucrose solution containing the indicated concentrations of digitonin (Sigma) or 1% Triton X-100. The reaction was stopped by adding trichloroacetic acid. To assess membrane association, mitochondria suspended in sucrose solution were sonicated on ice. Sonicated mitochondria were centrifuged at 4°C for 20 min at 100,000*g*, and the pellets were collected as the mitochondrial membrane fraction. The membrane fraction was further treated for 30 min on ice with 0.1 M Na_2CO_3 in sucrose solution, and separated into the supernatants and the membrane pellets.

Northern Blot Analysis—Total RNA was isolated from 6-month-old mouse (male) tissues using TRIzol

(Invitrogen) according to the manufacturer's instructions. Ten μg of the total RNA was separated on 1.2% agarose gels containing formaldehyde, and transferred to Hybond-N^+ (Amersham Biosciences). Labeling of the probe, hybridization, and detection of the signals were done using a DIG High Prime DNA labeling and detection system (Roche).

Construction of Anti-hM19 Antibody—pGEX-4T-3-hM19 or pQE32-hM19 was transformed into *Escherichia coli* BL21 strain. Expression of GST-hM19 and His-tagged hM19 was induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) at 22°C for 5 h, and the fusion proteins were affinity purified with glutathione-Sepharose 4B (Amersham Biosciences) and Ni-NTA His-Bind Resin (Novagen), respectively. The purified GST-hM19 was used to immunize two rabbits, and serum was collected and affinity purified with the recombinant His-hM19.

Western Blot Analysis—Samples were separated by electrophoresis on SDS–polyacrylamide gels (12% or 13.5% acrylamide) and then electrophoretically transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences). The membranes were probed with primary and horseradish peroxidase-conjugated secondary antibodies, and immunoreactive bands were visualized with enhanced chemiluminescence reagents (Amersham Biosciences). The following primary antibodies were used: anti-M19 (1 : 100, this study), anti-Porin (1 : 2000, Calbiochem), anti-OPA1 (1 : 1000) (*14*), anti-PDHE2 (1 : 1000, Molecular Probes), anti-PHB1 (1 : 200, NeoMarkers), anti-PHB2 (1 : 1000, Upstate Biotechnology), anti-TFAM (1 : 100, Santa Cruz Biotechnology), anti-mtSSB (1 : 2000) (*17*), anti-cytochrome *c* (1 : 100, BD Pharmingen), anti-p32 (1 : 200, BD Biosciences), anti-E2F-1 (1 : 50, Santa Cruz Biotechnology) and anti-Hax-1 (1 : 200, BD Transduction Laboratories).

Immunoprecipitation—Mitochondria purified from HeLa cells were suspended with radioimmune precipitation assay (RIPA) buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% sodium deoxycholate and 1% Triton X-100). After sonication for 4 min on ice, the solution was centrifuged at 4°C for 15 min at 10,000*g*. Five micrograms of anti-M19 antibody (described above) and protein G-Sepharose were added to the supernatant, and mixed at 4°C overnight on a rotating wheel. After washing three times, the immunoprecipitated complexes were separated by 13.5% SDS–PAGE, and stained with Coomassie brilliant blue (CBB).

Mass Spectrometry and Protein Identification—Protein bands on acrylamide gels were in-gel digested and mass-analysed as previously described (*14*).

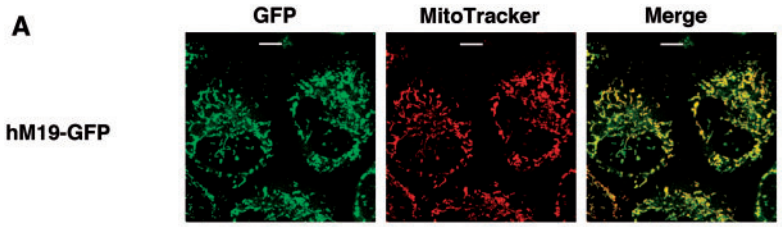
PCR Amplification of mtDNA Fragment—For amplification of a fragment of human mtDNA, the following primers were used: forward; 5′-CACAGCGCTAAGCTCG CACTG-3′ (4,527–4,547 nt), reverse; 5′-GCTAAGATTTTG CGTAGC-3′ (4,992–5,009 nt).

RESULTS AND DISCUSSION

A Novel Mitochondrial Protein M19—By our proteome analysis of human mitochondrial membrane protein from HeLa cells, an ~15 kDa protein, termed M19 was identified (data not shown, and manuscript in preparation). This protein is coded by the chromosome 6 open reading frame 125 (c6orf125, GenBank accession no. **BC006007**). The hM19 gene, located at 6p21.31, encodes 126 amino acids in a single open reading frame. When it was expressed in HeLa cells as a fusion protein with GFP at the C terminus, it was co-localized with mitochondria (Fig. 1A). M19 is an uncharacterized protein and possesses no homologous domain with other known proteins, although it is highly conserved among vertebrates (Fig. 1B). The homology is especially high in the amino-terminal region. The amino acid sequence of hM19 has 91% identity with that of the mouse homolog, and over 60% identity with the frog and zebra fish homologs. Although hM19-GFP is localized to mitochondria, its role as a possible mitochondrial target signal is predicted only with moderate to low probability, and its cleavage site is not predicted at all. The computer programs used for these prediction studies were Target P 1.1 (probability score, 0.423 for hM19 being a mitochondrial targeting signal) (*18*) and Mitoplot II (probability score, 0.2523) (*19*).

The tissue distribution pattern of M19 gene expression was examined by northern blotting. RNAs prepared from various mouse tissues were subjected to blot analysis using mouse M19 (mM19) coding sequence as a probe (Fig. 2). mM19 mRNA was detected as a single band of about 900 bp and was found in most tissues examined. Among them, the expression was especially abundant in brain, heart, skeletal muscle and kidney.

We established anti-hM19 rabbit-polyclonal antibody and used it to detect hM19 protein by western blotting in HeLa cell extracts. The Mitochondrial fraction and the PMFs were separated from HeLa cell cytoplasmic extracts by centrifugation, and probed with the antibody. An ~15 kDa band was detected in the mitochondrial fraction, but not in the PMF (Fig. 3A). The fractionation was controlled by the mitochondrial protein, Porin. The 15 kDa band decreased in intensity when endogenous hM19 was knockdowned by siRNA targeted to hM19 (Fig. 3A, siM19 lane). Thus, this protein band, detected only in the mitochondrial fraction, corresponds to hM19. Protein bands detected at over 75 kDa are believed to be non-specific bands because they were not affected by treatment with siM19 (Fig. 3A). A protein band with a mass of ~27 kDa, was also reduced in intensity in the siM19 lane (Fig. 3A). This band might be a form of post-translationally modified M19. To examine whether M19 exists in the nucleus, nuclear and cytoplasmic (the mitochondrial fraction plus PMF) fractions were probed with the antibody. M19 was faintly detected in the nuclear fraction, but majority of it in the cytoplasm (Fig. 3B). Although immunostaining of endogenous M19 by the antibody showed nuclear signals, they were not decreased with knockdown of M19 (Supplementary Fig. 1), indicating them as non-specific signals. Thus, M19 exists mainly in the mitochondria and, if any, few of it in the nucleus. We also confirmed that the anti-hM19 antibody cross-reacts with exogenously expressed mM19 (data not shown). Using this antibody, mouse tissue extracts were examined by western blotting. mM19 was detected in almost all the tissues tested, and was highly enriched in the brain (Fig. 3C).



B

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Hs  MAASRYRRFLKLCEEWPVDETKRGRDLGAYLRQRVAQAFREGENTQVAEPEACDQMYESL 60
Mm  MAALRYRRFLKLCEEWPVDETKRGRDLGAYLRQRVAQAFREGENTQIAEPEACDQMYESL 60
Rn  MAALRYRRFLKLCEEWPVDETKRGRDLGAYLRQRVAQAFREGENTQVAEPEACDQMYESL 60
Xl  MASLRYRRFLKLCEEWPVDETRVGRDLGAYIRQRVAQSFREGESTQVDDPDACDEMYASL 60
Gg  MAATRYRRFLKLCEEWPVEETKRQRDLGVFIRQRVAQAFREGENTQIADPETCDQMYESL 60
Dr  MSATRYRRFLKLCEEWPKDESKKGRDLGTFLRQRVASAFREGENTQISDPEKCDQMYESL 60
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Hs  ARLHSNYYKHKYPRPRDTSFSGLSLEEYKLILSTDTLEELKEIDKGMWKKLQEKFAPKGP 120
Mm  ARLHSNYYKHKYPRPRDTSFSGLSVEEYKLILSTDTLEEFQEMNKSMWKKLQEKFAPTRP 120
Rn  ARLHSNYYKHKYPRPRDTSFSGLSVEEYKLILSTDTLEEFQEMNKSVWRKLQEKFAPTRP 120
Xl  NRMNTNCYREKYPRLQDTSFTEVTAEEYRMVLASDNLKQMEDMKKGMWTRLREKFNVKPS 120
Gg  VRIHNNYYKNKYPRLKDTSFTGVTVQDCKMILATDILKQMEDMKKGTWRKLRERFYAKKS 120
Dr  ARINSNVYKEKFPRAKDTSFTGVTVEECRLLLATGSMQQTDEEKKGLWKTLMERFSSKPE 120
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Hs  EEDHKA---------- 126
Mm  EEKHKAWTRVLSRPRT 136
Rn  EEKHRAWTRVLSRPRT 136
Xl  EENN------------ 124
Gg  EEDSK----------- 125
Dr  DVPPEKAEK------- 129
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Fig. 1. **M19 is highly conserved among vertebrates.** (A) C-terminally GFP-tagged hM19 was expressed in HeLa cells. The cells were stained with the mitochondrial marker, MitoTracker Red. Images of the GFP-fusion protein (GFP) and red fluorescent signal (MitoTracker) were obtained by confocal microscopy, and were merged (Merge). Scale bars, 10 μm. (B) Alignment of the M19 amino acid sequences of *Homo sapiens* (Hs, **BC006007**), *Mus musculus* (Mm, **NM026063**), *Rattus norvegicus* (Rn, **XM342099**), *Xenopus laevis* (Xl, **NM001092076**), *Gallus gallus* (Gg, **XM418034**) and *Danio rerio* (Dr, **NM200758**). The alignment was done using Clustal W software (version 1.83, WWW Service at the European Bioinformatics Institute http://www.ebi.ac.uk/Tools/clustalw/). Asterisks indicate identical amino acids among the six species. The amino acids with conservative substitutions and semi-conservative substitutions based on physiochemical criteria are marked with *double dots* (:) and *single dots* (.), respectively.

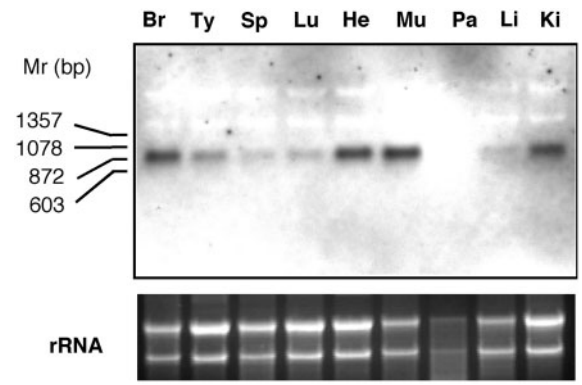

Fig. 2. **Expression of mouse M19 by northern blot analysis.** Total RNA (10 μg each) prepared from mouse (6-month-old) tissues was separated on agarose gels containing formaldehyde, and blotted onto a membrane. The RNA was probed with full-length mouse M19 coding region (top panel). The bottom panel shows ribosomal RNA (rRNA) stained with SYBR-Safe. The size markers are on the left. Br, brain; Ty, thymus; Sp, spleen; Lu, lung; He, heart; Mu, skeletal muscle; P_a, pancreas; Li, liver; Kd, kidney.

This pattern is similar to that observed for mRNA expression levels with the northern blots.

To further analyse the submitochondrial localization of M19, a protease-protection assay was performed. Mitochondria purified from HeLa cells were digested with trypsin in the presence or absence of digitonin or Triton-X 100. hM19 was decreased by trypsin treatment in the presence of digitonin and Triton-X 100, which disrupt both the inner and outer membranes (Fig. 3D, lane 5). However, hM19 was not decreased by trypsin in the presence of digitonin alone, which selectively disrupts the outer membrane (Fig. 3D, lanes 3 and 4). The digestion pattern of hM19 is similar to that of the matrix protein pyruvate dehydrogenase E2 (PDHE2), but not with that of the intermembrane space protein, optic atrophy 1 (OPA1) (Fig. 3D). Therefore, hM19 is localized in the matrix space of mitochondria. Next, the association of M19 with mitochondrial membranes was examined. When mitochondria isolated from HeLa cells were sonicated and treated with alkali solution after sonication, most of the M19 remained in the membrane pellets (ppt.) (Fig. 3E, lanes 3 and 5). This pattern is similar

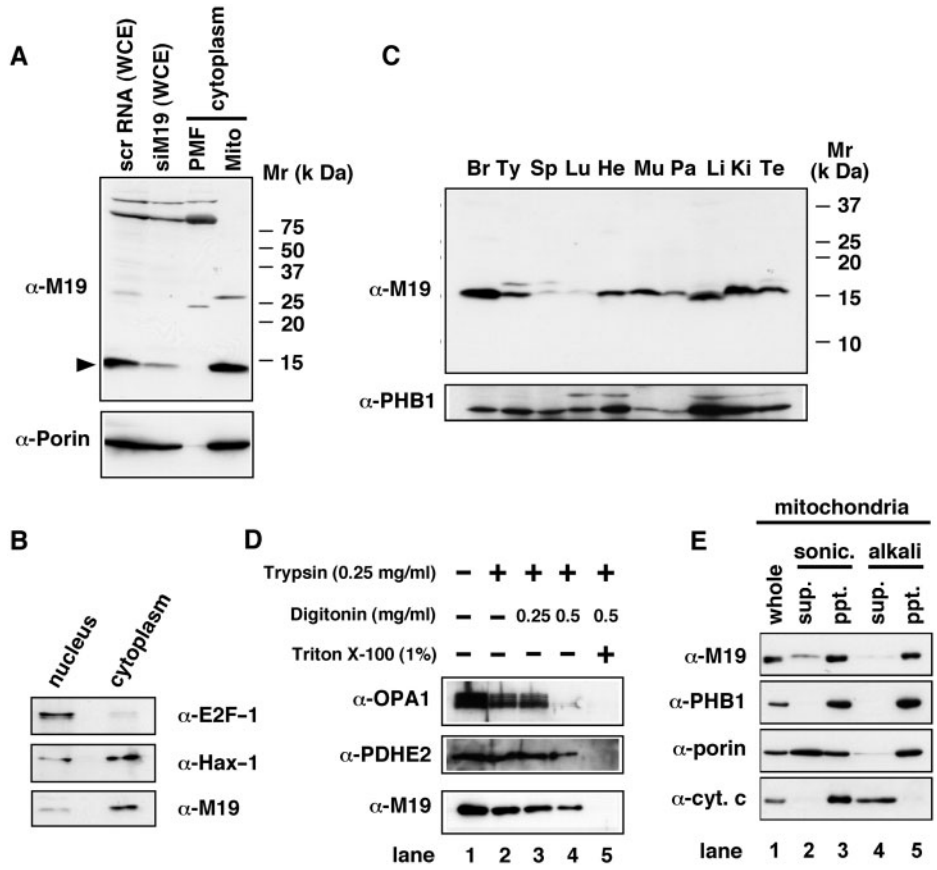

Fig. 3. **Distribution of mitochondrial M19 protein by western blot analysis.** (A) hM19 was detected in HeLa cell extracts by western blot analysis with anti-M19 antibodies. The whole-cell extracts (WCE) were prepared from HeLa cells transfected with scrambled RNA (scrRNA) or siRNA expression vector targeted to hM19 (siM19) at 5 days after transfection. HeLa cell cytoplasmic extracts were separated into a mitochondrial fraction (Mito) and PMF by centrifugation. These samples (100 μg of WCE and 50 μg of Mito and PMF) were probed with anti-M19 (upper panel, α-M19) and anti-Porin (lower panel, α-Porin) antibodies. The arrowhead shows specific hM19 protein bands. (B) Nuclear and cytoplasmic extract (50 μg each) were probed with anti-M19 antibody. E2F-1, a transcription factor, is nuclear marker, and mitochondrial Hax-1 is also detected. (C) The WCEs (100 μg each) prepared from male mouse (6-month-old) tissues were subjected to western blotting using anti-M19 or anti-PHB1 antibodies. The size markers are on the right. Br, brain; Ty, thymus; Sp, spleen; Lu, lung; He, heart; Mu, skeletal muscle; Pa, pancreas; Li, liver; Kd, kidney; Te, testis. (D) The protease-protection assay showed that hM19 is localized in the matrix space. Mitochondria were treated with trypsin, digitonin and/or Triton X-100, and analysed by western blotting using the indicated antibodies. (E) Mitochondria from HeLa cells were sonicated (*sonic.*) and separated into supernatant (*sup.*) and membrane pellets (*ppt.*) by centrifugation. The membrane pellets were further treated with Na_2CO_3 (*alkali*) and separated into '*sup.*' and '*ppt.*' by centrifugation. Samples were analysed by western blotting using the indicated antibodies.

to that of known membrane integrated proteins, PHB1 and Porin, but not to the membrane-associated protein cytochrome *c* (Fig. 3E, lane 4). Considering no transmembrane domain in M19, these results demonstrated that M19 is a mitochondrial matrix protein that tightly associates with inner membrane.

Association of M19 with Mitochondrial Nucleoids—mtDNA is known to exist in the mitochondrial matrix as a mtDNA-protein complex (mitochondrial nucleoids). mtDNA sublocalizes in the mitochondria, as demonstrated by the spotted signals observed with PicoGreen staining, and by immunohistochemistry using antibodies against DNA (*20*, *21*). We found that when a C-terminally FLAG-tagged hM19 (hM19-FLAG) was expressed in HeLa cells, it also sublocalized as spotted signals within mitochondria, similar to that observed for mtDNA (Fig. 4A). Actually, when hM19 was expressed in HeLa cells as a fusion protein with DsRed1 at the C terminus, it was partially co-localized with mtDNA that apparently stained with PicoGreen (Fig. 4B). These observations strongly suggest that M19 proteins are included in the nucleoid complex.

Next, to investigate whether the M19 protein is involved in mitochondrial nucleoids, we performed immunoprecipitation with an anti-M19 antibody in HeLa cell mitochondria, and analysed the co-precipitated proteins by LC-MS/MS (Fig. 4C). Not only mtSSB, but also LONP1 and PDIP38, which were recently reported to be putative mitochondrial nucleoid components in higher organisms (*12*, *13*), were identified as co-immunoprecipitated factors with M19 protein. Although we tried to detect the interaction by immunoblot analysis, we could not detect it clearly, possibly due to its weak interaction and the characteristics of the antibodies. To assess the association of M19 with the nucleoids, we fractionated the mitochondrial nucleoids

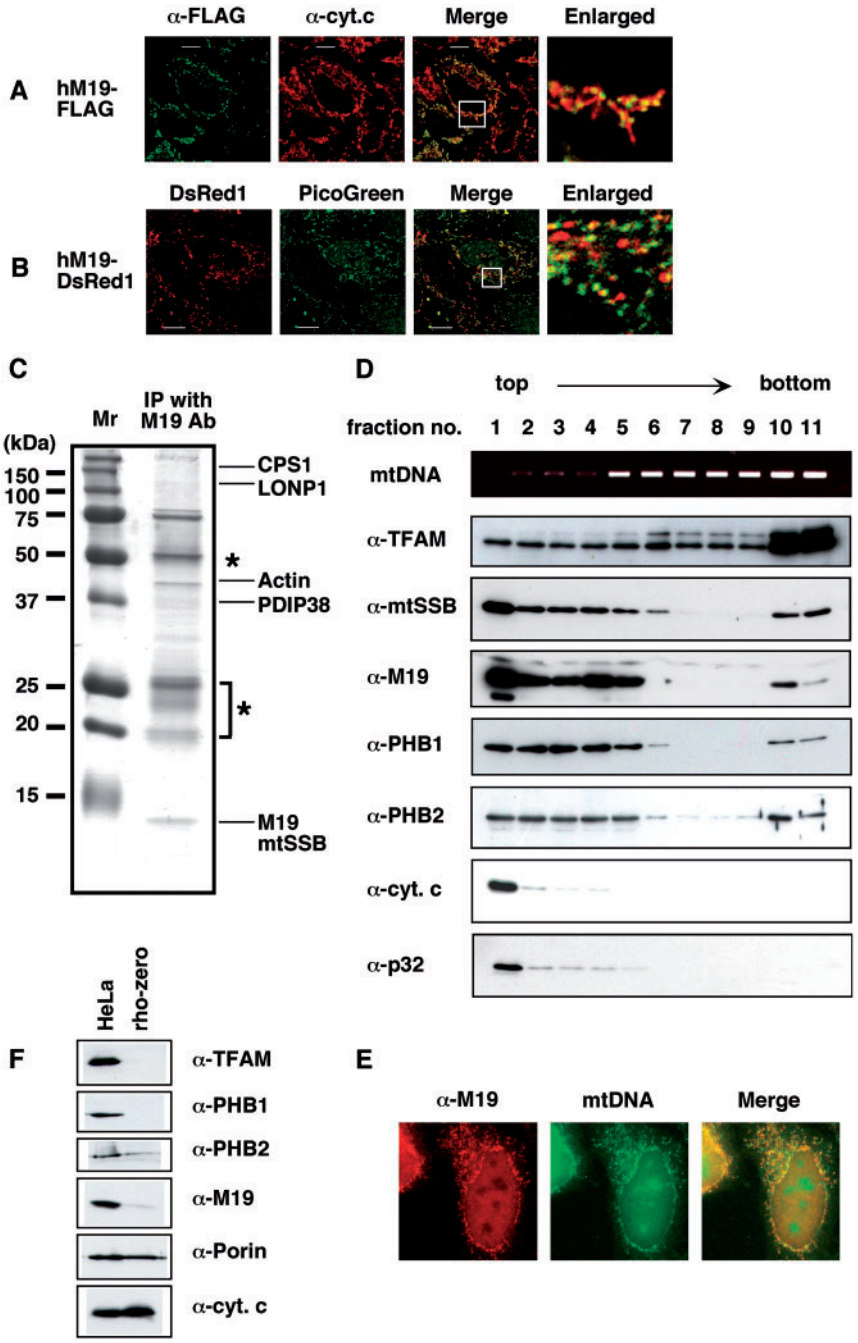

Fig. 4. **Interaction of M19 protein with mitochondrial nucleoids.** (A and B) C-terminally FLAG-tagged hM19 proteins (A) and C-terminally DsRed1-tagged hM19 (B) were expressed in HeLa cells. The HeLa cells were fixed and immunostained with anti-FLAG (α-FLAG) and anti-cytochrome c (α-cyt. c) antibodies (A), and were stained with PicoGreen (B). These images were merged (Merge), and the square boxes represent the area enlarged (Enlarged). Scale Bars, 10 μm. (C) Proteins extracted from HeLa cell mitochondria were immunoprecipitated with an anti-M19 antibody. Precipitated proteins were separated by SDS–PAGE, and stained with CBB. The bands were cut out and identified by mass spectrometry. Protein names are shown on the right. Asterisks represent heavy and light chains of the antibody, and molecular size markers are shown on the left. (D) Mitoplasts were lysed in Triton buffer, and the extracts were subfractionated by velocity centrifugation on a step-wise glycerol gradient. Fractions were collected from the top of the gradient, and numbered. Nucleic acids were extracted from a portion of each fraction using a standard phenol–chloroform protocol, and were subjected to PCR amplification of the fragment of mtDNA (mtDNA). Each fraction was also subjected to western blotting using the indicated antibodies. (E) Combined immunofluorescence and FISH. HeLa cells were probed with anti-M19 antibody and mtDNA probe, sequentially. (F) WCEs were prepared from HeLa cells or ρ^0 HeLa cells, and were subjected to western blotting using the indicated antibodies.

from HeLa cells using velocity sedimentation of mitochondrial mitoplasts in a glycerol gradient. After centrifugation, fractions were collected from the top of the gradient. mtDNA showed a broad distribution, and its signals gradually increased from top to bottom (Fig. 4D). TFAM, a well characterized nucleoid factor, also showed a broad distribution, indicating that various forms of nucleoids exists along different sedimentation velocity (Fig. 4D), as described by another group (*13*). In their experiment, mitochondrial nucleoids in HeLa cells produced heterogeneous sedimentation profiles, and were separated into rapidly and slowly sedimenting fractions. When our fractions were probed with anti-hM19, hM19 was detected in both slowly sedimented (lane 5) and rapidly sedimented (lane 10, 11) fractions; the latter is the TFAM-enriched fraction (Fig. 4D). In addition, the fractionation pattern of hM19 was similar to that of known nucleoid factor mtSSB and those of PHB1 and PHB2, which were recently identified as mitochondrial nucleoid components of HeLa cells (*12*, *13*), but not to that of cytochrome *c* and p32 as unrelated protein for mitochondrial nucleoids (Fig. 4D). Therefore, it is likely that hM19 is a component of mitochondrial nucleoids.

We further examined the distribution of endogenous M19 by combined immunofluorescence and FISH technologies (*22*). HeLa cells were fixed and immunostained with anti-M19 antibody. Then, the cells were fixed again and probed with mtDNA probes. Signals of M19 appeared spotted in the cytoplasm, which are detected on the mitochondria and disappear with knockdown of M19 (Supplementary Figs 1 and 2). A part of endogenous M19 signals overlapped with mtDNA signals, well in accordance with the above nucleoid fractionation (Fig. 4E).

In another experiment, we observed that the expression levels of the nucleoid proteins, TFAM, PHB1 and PHB2, decreased in ρ^0 HeLa cells (Fig. 4F), which are cells lacking mtDNA. Expression of TFAM is closely related to the amount of mtDNA (*7*). It has also been reported that the expression level of TFAM is reduced in A549 ρ^0 cells (*11*), suggesting a down-regulation of some nucleoid proteins in these cells. The present results showing that M19 is also decreased in ρ^0 HeLa cells (Fig. 4F) strongly support the association of M19 with the nucleoids.

In the present study, we identified a novel mitochondrial protein, M19, and demonstrated its interaction with mitochondrial nucleoids. What is the role of M19 in the nucleoids? In a recent study, we found that the nucleoid component PHB1 acts to maintain the organization and stability of the mitochondrial nucleoids (*23*). Knockdown of PHB1 reduced the staining of mtDNA by fluorescent dyes, such as ethidium bromide (EtBr) and PicoGreen, due possibly to the altered organization of nucleoids. In addition, the knockdown of PHB1 reduced mtDNA levels through TFAM down-regulation. When we performed RNAi-mediated knockdown of hM19 in HeLa cells, these phenotypes were not observed (data not shown), suggesting that M19 is involved in the maintenance of mtDNA in a different manner from known nucleoid component, such as PHB1 and TFAM. In general, behaviour of mtDNA tends to be different among many culturing cells and tissues. For example, it seems likely that accumulation of deleted mtDNA occurs in tissues containing non-dividing cells, such as heart and brain, rather than in actively dividing cultured cells (*24*). Therefore, future knockout study will provide the nucleoid function of M19 in these tissues. Very recently, Bogenhagen *et al*. (*25*) represents a model for a layered structure of mtDNA nucleoids, in which the nucleoids are separated into the central core region that tightly binds to mtDNA and the peripheral region surrounding it. In their study, TFAM is included in the central core region, whereas PHB proteins (PHBs) are in the peripheral region because PHBs are not crosslinked to mtDNA. Considering that the fractionated pattern of the hM19 in the nucleoids is similar to those of PHBs but not to that of TFAM (Fig. 4D), M19 might also be included in the peripheral region and be preferentially involved in the translation of mtDNA or respiratory complex assembly rather than the transcription and replication of mtDNA. Mitochondrial nucleoids are associated with the inner membrane by unknown tethering factors. M19 might be one of these tethering factors, since it strongly associates with the mitochondrial membrane (Fig. 3D). It will be an exciting task in the future to analyse the physiological function of M19 in the metabolism of mtDNA as a component of mitochondolial nucleoids.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared

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