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PDIP38 Associates with Proteins Constituting the Mitochondrial DNA Nucleoid

Xiaoli Cheng[1,2], Tomotake Kanki[1], Atsushi Fukuoh[1], Kippei Ohgaki[1], Ryu Takeya[3], Yoshimasa Aoki[1], Naotaka Hamasaki[1] and Dongchon Kang[1,*]

[1]Department of Clinical Chemistry and Laboratory Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka 812-8582; [2]Basic Medical College, Zhengzhou University, Henan, China; and [3]Department of Biochemistry and Biotechnology, Kyushu University, Fukuoka 812-8582

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Human mitochondrial DNA takes on a large protein-DNA complex called a nucleoid or mitochromosome. Mitochondrial transcription factor A (TFAM) is a major component of the complex. During an attempt to search for proteins associated with the TFAM-containing complex by a proteomic method, we found one protein that has not been considered to be mitochondrial: PDIP38. PDIP38 was initially identified as a binding protein to nuclear DNA polymerase δ. PDIP38 is almost exclusively recovered from the mitochondrial fraction of human HeLa cells. PDIP38 is completely cleaved when TritonX-100–solubilized mitochondria are treated with proteinase K, but not when mitoplasts devoid of outer membranes are treated, indicating that PDIP38 is located in the mitochondrial matrix. TFAM and mitochondrial single-stranded DNA binding protein (mtSSB) are co-immunoprecipitated with PDIP38 by anti-PDIP38 antibodies. On the other hand, only the latter is crosslinked to PDIP38 when mitochondria are treated with a crosslinker, formaldehyde. In addition to mtSSB, 60 kDa heat shock protein and a Lon protease homolog, both of which have single-stranded DNA binding activity, are also crosslinked. PDIP38 associates with the nucleoid components and could be involved in the metabolism of mitochondrial DNA.

Key words: mitochondria, mitochondrial DNA, mitochondrial transcription factor A (TFAM), nucleoid, proteomics.

Human mitochondrial DNA (mtDNA) is a 16.5-kb double-stranded circular molecule that encodes 13 essential subunits of the mitochondrial respiratory chain, 22 tRNAs, and 2 rRNAs. There are hundreds to thousands of copies of mtDNA in one cell. The maintenance of mtDNA integrity is essential for the normal function of the respiratory chain, and so for the normal living of individuals.

Mitochondrial transcription factor A (TFAM), a transcription factor for mtDNA, enhances mtDNA transcription in a promoter-specific fashion in the presence of mitochondrial RNA polymerase and transcription factor B (*1*). TFAM possesses DNA-binding properties regardless of sequence specificity (*2*), although it shows a higher affinity for the light and heavy strand promoters (LSP and HSP, respectively).

Mitochondrial DNA takes on a large protein-DNA complex called a nucleoid or mitochromosome (*3*). Proteomic characterization of protein-mtDNA complexes has been done in *Saccharomyces cerevisiae* (*4*) and *Xenopus* oocytes (*5*). In *S. cerevisiae* Abf2p, a structural TFAM homolog is detected as a main component of the nucleoid, and appears to function in the maintenance of mtDNA and the nucleoid structure (*6*). Recent reports also suggest the existence of such a higher structure of mtDNA in mammals (*7*–*11*). Because TFAM is as abundant as Abf2p in mitochondria (*10*), TFAM is also believed to play a major role in the maintenance of the nucleoid structure. In fact, the expression of human TFAM in the yeast *abf2* strain rescues the phenotype, implying a potential functional homology between human TFAM and yeast Abf2p (*12*). We have established stable and inducible human cell lines overexpressing TFAM, and have found that the amount of TFAM is strictly correlated with the amount of mtDNA (*11*), suggesting that the nucleoid structure is also essential for maintaining mammalian mtDNA.

Despite of its importance, little has been elucidated about the structure of the mammalian mtDNA nucleoid. During efforts to identify protein components of the nucleoid, we found one protein, PDIP38, that has not previously been considered to be a mitochondrial protein. PDIP38 was initially identified as a protein binding to nuclear DNA polymerase δ and proliferating cell nuclear antigen (*13*). Its physiological function mostly remains to be elucidated. Here we report that PDIP38 localizes exclusively in the mitochondrial matrix, and is crosslinked to proteins that bind to single-stranded DNA.

MATERIALS AND METHODS

Materials—Anti-mtSSB (*10*), anti-DNA polymerase γ large subunit (*14*), anti-TFAM (*10*), and anti-endoG antibodies (*15*) were described previously. Anti-FLAG, anti-HA, anti-calnexin, anti-histone antibodies were purchased from Sigma, Covance, StressGen, and Upstate,

*To whom correspondence should be addressed. Tel: +81-92-642-5749, Fax: +81-92-642-5772, E-mail: kang@mailserver.med.kyushu-u.ac.jp

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respectively. MitoTracker Red CMXRos was from Molecular Probes.

Predicting the Possibility of Mitochondrial Localization and the Mitochondrial Targeting Signal—The MitoProt II program (http://ihg.gsf.de/mitop2/start.jsp) was used to predict the possibility of mitochondrial localization and the mitochondrial targeting signal (*16*).

Expression of PDIP38 in HeLa tet-off Cells—cDNA of PDIP38 containing the deduced first methionine site was amplified from a cDNA library of human HeLa cells by polymerase chain reaction (PCR) using a primer set: 5′-ATG GCA GCC TGT ACA GCC CGG CGG GCC CTG and 5′-CTA CCA GTG AAG GCC TGA GGG TGG TGT CTT. Then *Bam*HI and *Spe*I sites were added to the 5′- and 3′-terminals, respectively, of the cDNA by a second PCR using primers, 5′-ATA TGG ATC CAT GGC AGC CTG TAC AGC CCG and 5′-ATATACTAGTCC AGT GAA GGC CTG AGG GTG. The PCR product was digested with *Bam*HI and *Spe*I. The DNA fragment encoding PDIP38, a DNA fragment encoding an HA-tag (see Ref. *11*) and a pTRE2hyg vector (Clontec) digested with *Bam*HI and *Nhe*I were ligated. The vector was named pp38HA. We transfected HeLa tet-off cells with the pp38HA vector and selected cells bearing the transgene in the presence of G418 (400 μg/ml) and hygromycin B (200 μg/ml) as previously described (*11*).

Immunofluorescent Imaging of HeLa Cells—Human HeLa cells were incubated in the presence of 100 nM MitoTracker Red CMXRos for 20 min. After washing with phosphate-buffered saline (PBS) three times, the cells were fixed with acetone/methanol (=50:50, v/v) for 5 min. After washing with PBS three times, the fixed cells were blocked with PBS containing 1% bovine serum albumin (BSA) (PBS/BSA) for 30 min. Then the cells were incubated with 250-fold diluted anti-HA antibody in PBS/BSA for 1 h. After washing the cells with wash buffer (PBS containing 0.1% Tween-20) three times, the cells were incubated with 250-fold diluted Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes) for 30 min. Fluorescence images were obtained under a confocal laser microscope (Carl Zeiss).

Expression of the Recombinant PDIP38 Protein—DNA encoding mature PDIP38 (from Gly 59) was amplified by PCR using *Bam*HI and *Xho*I sites-containing primers (5′-ATA TGG ATC CGG CAA AGT GTT GGA GAC AGT and 5′-ATA TCT CGA GCT ACC AGT GAA GGC CTG AGG). The amplified DNA was digested with *Bam*HI and *Xho*I, and then inserted between the same sites of pPRO-EX-HTb, an expression vector for His-tagged proteins, and pGEX-4T-1, an expression vector for GST-tagged proteins, respectively. The recombinant mature PDIP38 protein with an N-terminal His-tag (His-PDIP38) was expressed in *Escherichia coli* BL21 cells at 37°C. More than 90% of His-PDIP38 was recovered from the insoluble fraction, but a few percent was recovered from the soluble fraction. We purified the His-PDIP38 remaining in the soluble fraction using a Ni-resin column. Recombinant mature PDIP38 with an N-terminal GST-tag (GST-PDIP38) was also mostly recovered from the insoluble fraction. GST-PDIP38 in the insoluble fraction was separated by electrophoresis in a polyacrylamide gel containing sodium dodecylsulfate (SDS-PAGE), and then extracted from the gel. The purified GST-PDIP38 protein was used to produce rabbit anti-PDIP38 antibodies.

Cell Fractionation and Intracellular Localization—HeLa cells cultured in 10-cm dishes were scraped off with a cell lifter (Costar), suspended in PBS, precipitated by centrifugation, and washed with homogenizing buffer (10 mM HEPES-KOH, pH 7.4, 0.25 M sucrose, and 1 mM EDTA). The cells were then suspended in 4 volumes of the same buffer, homogenized with a Potter-Elvehjem homogenizer, and centrifuged at 900 × *g* for 10 min. We collected the pellet as the nuclear fraction. The supernatant was centrifuged at 10,000 × *g* for 6 min. The second pellet was collected as the crude mitochondrial fraction (see below for a more purified mitochondrial fraction). The second supernatant was centrifuged at 210,000 × *g* for 10 min. The third pellet was collected as the microsomal fraction. The third supernatant was collected as the cytosolic fraction. The protein concentration of each fraction was measured using a 2-D quant Kit (Amersham biosciences). Twenty micrograms of protein of each fraction was separated by 12% SDS-PAGE and analyzed by immunoblotting.

Submitochondrial Localization—The crude mitochondria were suspended in hypotonic buffer (10 mM HEPES-KOH, pH 7.4, and 1 mM EDTA) at 4°C for disruption of the mitochondrial outer membranes. After the hypotonic treatment, the mitochondria were precipitated by centrifugation at 10,000 × *g* for 6 min. The mitochondria were resuspended in hypotonic buffer and then digested with proteinase K (200 μg/ml) with or without 1% Triton X-100 on ice for 20 min. The protein was precipitated by the addition of 15% trichloroacetic acid, and the precipitates were solubilized in sample buffer (6% SDS, 150 mM Tris-base, 10 mM EDTA, and 25% glycerol), separated by SDS-PAGE, and analyzed by immunoblotting.

Immunoprecipitation Using Anti-PDIP38 Antibodies—Each step was done at 4°C or on ice. Jurkat cells were homogenized with a Potter-Elvehjem homogenizer in 2 ml of homogenizing buffer and centrifuged at 900 × *g* for 10 min. The supernatant (about 2 ml) was diluted with 2 ml of 20% Percoll buffer [0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 20% Percoll (Amersham Bioscience)], and the sample was overlaid on a discontinuous Percoll density gradient (4 ml of 40% and 4 ml of 20% Percoll buffer) in a 12-ml centrifugation tube. After centrifugation at 24,000 rpm for 1 h using an SW41Ti rotor (Beckman), a mitochondrial band located in the middle of the tube was taken. Two to 3 mg of mitochondrial protein were solubilized in 1 ml of IP buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% BSA, and 0.5% NP-40), and then 40 μl of magnetic beads were coated with anti-PDIP38 antibodies (anti-PDIP38 antibodies were immobilized on the magnetic beads according to the manufacturer's instructions: tosylactivated Dynabeads M-280, Dynal). After 2 h rotation, the beads were washed four times with wash buffer (the IP buffer without BSA), suspended in SDS buffer (10 mM Tris-HCl, pH 7.4 and 1% SDS), and heated at 95°C for 3 min. The eluted proteins were separated by 12% SDS-PAGE and analyzed by immunoblotting.

DNA Polymerase γ—A His-tagged DNA polymerase γ large subunit and a FLAG-tagged small subunit were co-expressed in sf9 cells. The recombinant protein complex was purified with Ni^{+}-resins. Two micrograms of DNA polymerase γ were mixed with 1 μg of PDIP38 protein in 1 ml of PD buffer containing 10 mM Tris-HCl, pH 7.6,

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150 mM NaCl, 0.5% NP-40, and 1× of a protease inhibitor mix (Complete mini, Roche). After 2-h incubation at 4°C and another 2-h incubation with anti-PDIP38 antibodies, PDIP38 was precipitated by adding 120 μl of 20% protein A Sepharose 4B (Amersham Bioscience). The resin was washed with 1 ml of PD buffer 3 times, and the proteins were eluted with SDS buffer, separated by 8 and 12% SDS-PAGE, and analyzed by immunoblotting.

Immunoprecipitation after Crosslinking—All procedures were done at 4°C or on ice. Two milligrams of mitochondrial protein were suspended in 1 ml of crosslinking buffer (0.25 M sucrose, 20 mM HEPES-KOH, pH 7.4, 2 mM EDTA, and 25 mM NaCl). Then 27 μl of 37% formaldehyde or distilled water (as a control) were added, and the mixture was kept on ice for 2 h. After incubation, 125 μl of 1 M glycine was added to quench the crosslinking reaction, and the sample was centrifuged at 10,000 × *g* for 1 min to precipitate the mitochondria. The mitochondria were solubilized with 100 μl of the IP buffer containing 1% SDS. After 10-fold dilution with 1 ml of IP buffer, the immunoprecipitation reaction was done as described above. The immunoprecipitates were heated at 95°C for 30 min to cleave the crosslinks, and the proteins were separated by 12% SDS-PAGE. Bands were visualized by staining with Coomassie Brilliant Blue (CBB).

Surface Plasmon Resonance Assay—The instrument used was the BIACORE 1000 (Biacore International AB, Uppsala, Sweden). The analyte protein was dialyzed against PBS and diluted in running buffer (PBS containing 0.005% Tween-20) before use to the concentration indicated in the figure. The same amount of 5′-biotinylated single-stranded DNA (biotin-5′-GGTCGAGGAATTCGCCTTCTGC-3′) and double-stranded DNA were injected and immobilized on streptavidin-coated SA sensor chips (Biacore). The double-stranded DNA was prepared by annealing the biotinylated single-stranded DNA with its complementary DNA (5′-GCAGAAGGCGAATTCCTCGACC-3′). All binding and dissociation reactions were performed in running buffer flowing at 20 μl/min on the sensor ships. The dissociation constant (K_d) was estimated with BIA-evaluation Software 3.1.

In-Gel Protein Digestion and LC-MS/MS—A CBB-stained band was excised and the protein was digested with Lysyl Endopeptidase (WAKO, Japan) in digestion buffer (100 mM Tris-HCl, pH 9.0, 0.5% SDS, and 1 mM EDTA) for 12 h. The supernatant containing the digested peptides was analyzed by liquid chromatography and mass spectrometry (LC/MS/MS) (Agilent 1100 Series an LC/MS system). The peptide masses were assigned to the peptide mass databases using the MASCOT program (MATRIX SCIENCE). All proteins in the SWISS-PROT mammalian database were taken into account. The peptide mass error was limited to 1.0 Da and the MS/MS mass error was limited to 0.8 Da. Only one missed cleavage was accepted.

RESULTS AND DISCUSSION

PDIP38 in the Mitochondrial Matrix—PDIP38 is a protein composed of 368 amino acids as deduced from its cDNA. The existence of a 59-amino acid N-terminal mitochondria targeting sequence was predicted by MitoProt II, a prediction program for mitochondrial localization. The probability of mitochondrial localization is 0.9998 for

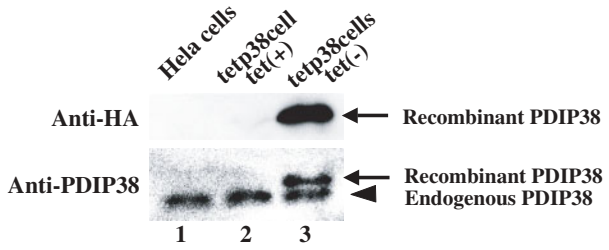

Fig. 1. **Inducible expression of recombinant PDIP38 with an HA tag.** PDIP38 with an HA tag was inducibly expressed in HeLa cells using a tet-off system as described under "MATERIALS AND METHODS." PDIP38 proteins were detected by Immunoblotting with anti-HA and anti-PDIP38 antibodies. Lane 1: no transfection; lane 2, tet (+), *i.e.* the pp38HA-transfected cells were cultured in the presence of doxycyclin; lane 3, tet (–), *i.e.* the pp38HA-transfected cells were cultured in the absence of doxycyclin. Arrows and the arrowhead indicate recombinant and endogenous PDIP38, respectively.

PDIP38. Then, we produced stable cell lines that express the recombinant full length PDIP38 with a C-terminal HA tag (PDIP38-HA) upon induction by doxycycline-withdrawal. The recombinant PDIP38-HA was seen only after the induction by doxycycline-withdrawal (Fig. 1, lane3 in upper and lower panels). In immunocytochemistry, the PDIP38-HA was granularly stained with anti-HA antibodies after (Fig. 2, middle in a lower panel) but not before (Fig. 2, middle in an upper panel) induction. The HA-staining is completely co-localized with the mitochondria visualized with a MitoTracker dye, suggesting that PDIP38 has a mitochondria targeting signal.

Then we examined the intracellular localization of endogenous PDIP38. We prepared four fractions from HeLa cells, nuclear, mitochondrial, microsomal, and cytosolic. The mitochondrial fraction contained essentially no histone, a nuclear protein, indicating minimal contamination of the mitochondrial fraction with nuclei (Fig. 3, a second panel). PDIP38, as well as TFAM, was found mostly in the mitochondrial fraction (Fig. 3, third and fourth panels); both were also very weakly detected in the nuclear fraction (Fig. 3, lane 1 in third and fourth panels). We could not deny at present that a small part of the two proteins exist in the nuclei. Thus we do not exclude the possibility that PDIP38 is bound to DNA polymerase δ *in vivo* (*13*).

Next we examined the submitochondrial localization of PDIP38. The mitochondrial outer membranes were disrupted by hypotonic treatment (Fig. 4, lanes 3 and 4) as verified by the observation that endonuclease G, which is localized in the intermembrane space, was completely digested by proteinase K (Fig. 4, lane 4 in a first panel). On the other hand, TFAM, which localizes in the matrix, was resistant to proteinase K digestion (Fig. 4, lane 4 in a middle panel), indicating that the inner membranes were intact. Under these hypotonic conditions, PDIP38 was not cleaved by proteinase K (Fig. 4, lane 4 in a lowest panel). When the inner membranes were solubilized with a non-ionic detergent, Triton X-100, both TFAM and PDIP38 were cleaved by proteinase K (Fig. 4, lane 5 in middle and lowest panels). Taken together, these results suggest that PDIP38 is localized in the mitochondrial matrix. Very recently, PDIP38 has been reported to be localized in

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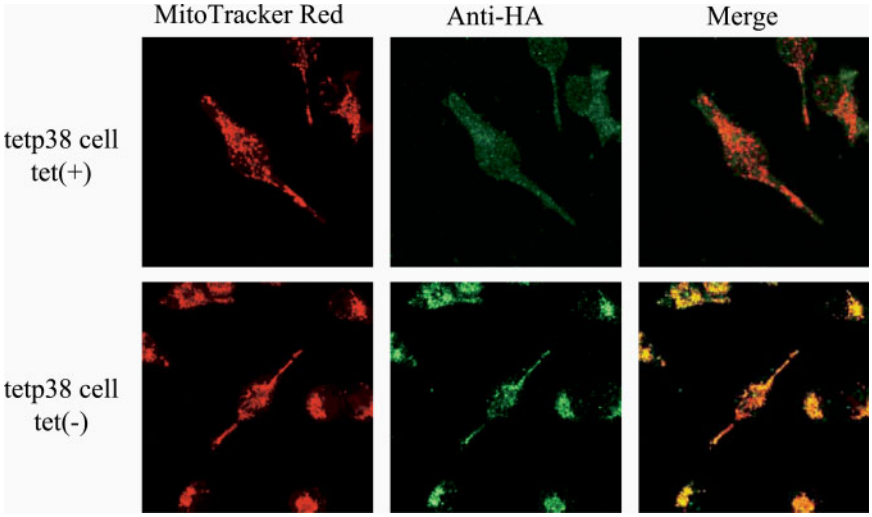

Fig. 2. **Immunocytochemistry of recombinant PDIP38.** The pp38HA-transfected cells were cultured in the presence (upper panels) or absence (lower panels) of doxycyclin. Mitochondria and the recombinant PDIP38 were visualized with a mitochondria-staining dye, MitoTracker red, (left panels) and anti-HA antibodies (middle panels), respectively. The left and middle panels are merged in the right panels.

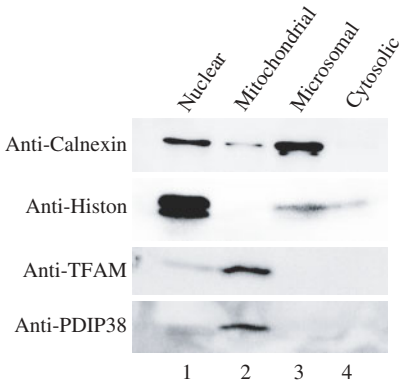

Fig. 3. **Subcellular localization of endogenous PDIP38.** The HeLa cells were separated into nuclear (lane 1), mitochondrial (lane 2), microsomal (lane 3), and cytosolic (lane 4) fractions. The indicated proteins were detected by immunoblotting. Calnexin, histone, and TFAM were used as markers for endoplasmic reticulum, nuclei, and mitochondria, respectively.

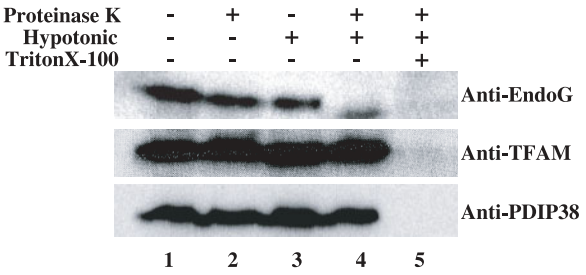

Fig. 4. **Submitochondrial localization of PDIP38.** Mitochondria were incubated in hypotonic buffer for disruption of the outer membranes without (lanes 3 and 4) or with a nonionic detergent, Triton X-100 (lane 5). Then the mitochondria were digested with proteinase K (lanes 2, 4, and 5). The indicated proteins were detected by immunoblotting. Endonuclease G (endoG) and TFAM are markers for the mitochondrial intermembrane space and matrix, respectively.

mitochondria, although localization in the matrix was not addressed (*17*).

Proteins Associated with PDIP38—PDIP38 was originally identified as a protein that binds to DNA polymerase δ and proliferating cell nuclear antigen (*13*), and so is expected to be involved in DNA metabolism, such as replication or repair. We immunoprecipitated NP-40–solubilized mitochondria with anti-PDIP38 antibodies, and found TFAM and mitochondrial single-stranded DNA binding protein (mtSSB) (Fig. 5). Conversely, PDIP38 was hardly co-immunoprecipitated with anti-TFAM (results not shown), which raises the possibility that only a very small part of PDIP38 may associate with TFAM. DNA polymerase γ, a mitochondrial DNA replicating DNA polymerase, was not detected in the immunoprecipitates (Fig. 5). That there is no association between DNA polymerase γ and PDIP38 was further confirmed using purified recombinant DNA polymerase γ and PDIP38. Neither the large subunit (catalytic subunit) nor the small subunit (accessory subunit) was coimmunoprecipitated together with PDIP38 by anti-PDIP38 antibodies (Fig. 6).

To look further for proteins associated with PDIP38, we crosslinked mitochondrial proteins by treating

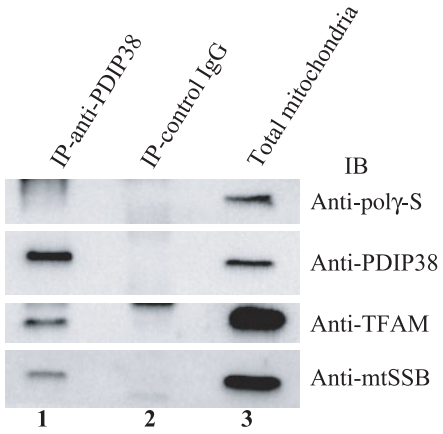

Fig. 5. **Immunoprecipitation of mitochondrial lysates with anti-PDIP38 antibodies.** Mitochondria solubilized with a nonioninc detergent, NP-40 were subjected to immunoprecipitation (IP) with anti-PDIP38. A half equivalent amount was applied in lane 3 for immunoblotting (IB). Lane 1, immunoprecipitates with anti-PDIP38; lane 2, immunoprecipitates with control IgG; lane 3, solubilized total mitochondria.

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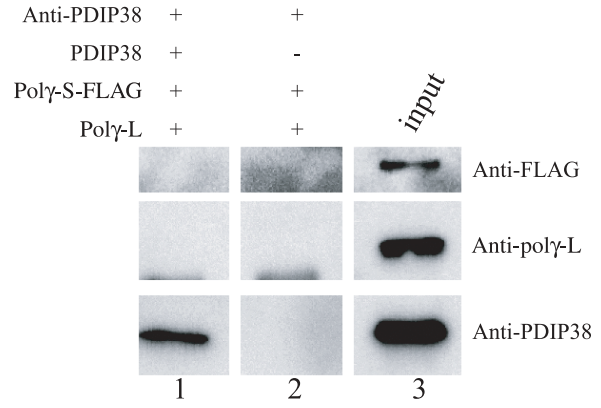

Fig. 6. **PDIP38 is not associated with DNA polymerase γ.** Purified recombinant PDIP38, a DNA polymerase γ small subunit (polγ-S) with a FLAG tag, and a DNA polymerase γ large subunit (polγ-L) with a His tag were incubated and immunoprecipitated with anti-PDIP38 antibodies. An equivalent amount was applied in each lane. Proteins were detected by immunoblotting using the indicated antibodies.

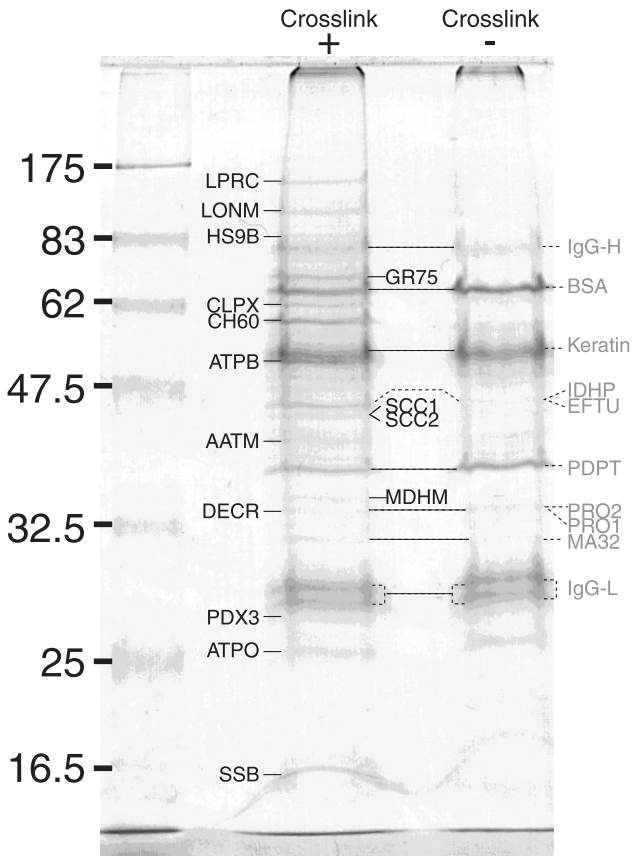

Fig. 7. **Mitochondrial proteins crosslinked with PDIP38.** Mitochondria were treated with formaldehyde to crosslink the proteins. Then the crosslinked or non crosslinked mitochondria were solubilized with 1% SDS, diluted fivefold, and immunoprecipitated with anti-PDIP38 antibodies. The immunoprecipitated proteins were separated by SDS-PAGE, and then each band stained with CBB was identified by LC/MS/MS as described under "MATERIALS AND METHODS." See Table 1 for protein names. Proteins expressed in shaded letters were detected under both conditions. BSA, which was used as a blocking reagent, was artificially released from the beads. Keratin may have contaminated the gel during SDS-PAGE.

Table 1. **Proteins detected only after crosslinking treatment.**

Abbreviation	Full name
LPRC	130 kDa leucine-rich protein
LONM	Lon protease homolog
HS9B	Heat shock protein HSP 90-beta
GR75	Stress-70 protein, mitochondrial
CLPX	ATP-dependent Clp protease ATP-binding subunit
CH60	60 kDa heat shock protein
ATPB	ATP synthase beta chain
SCC1	Squamous cell carcinoma antigen 1
SCC2	Squamous cell carcinoma antigen 2
AATM	Aspartate aminotransferase
MDHM	Malate dehydrogenase
DECR	2,4-dienoyl-CoA reductase
PDX3	Thioredoxin-dependent peroxide reductase
ATPO	ATP synthase oligomycin sensitivity conferral protein
SSB	Single-stranded DNA-binding protein

The proteins listed were detected only when proteins were crosslinked as shown in Fig. 7.

mitochondria with formaldehyde. After crosslinking with formaldehyde, the mitochondria were sonicated, extensively solubilized with 1% SDS, diluted, and then immunoprecipitated with anti-PDIP38 antibodies. Under these conditions, mtSSB, but not TFAM, was detected in the immunoprecipitates (Fig. 7), suggesting that PDIP38 is located close to mtSSB, and that the apparent association of PDIP38 with TFAM (Fig. 5) might be very unstable or indirect.

The other crosslinked proteins are listed in Table 1. A majority of them were also found in the mtDNA nucleoids that are prepared by immunoprecipitation with anti-TFAM antibodies (results not shown). In the context of single-stranded DNA binding of mtSSB, we first took note of the mitochondrial 60 kDa heat shock protein (CH60). The yeast 60 kDa heat shock protein (HSP60) is known to bind to DNA (*4*). Human CH60 has the ability to bind to single-stranded DNA with a K_d of about 1.0 μM (results not shown). Likewise, it may be significant that a Lon protease homolog is detected. It has been reported that the Lon protease homolog preferentially binds to single-stranded DNA with a sequence of the D-loop region, which is a main regulatory element for replication and transcription. In addition, this binding enhances the protease activity of the homolog (*18*). Thus its single-stranded DNA-binding activity is thought to play a role in mtDNA metabolism. Because we could not detect the binding of the recombinant PDIP38 to single-stranded DNA or to double-stranded DNA by the Biacore system (results not shown), the observed crosslinking of PDIP38 with the three single-stranded DNA binding proteins may not take place *via* DNA. It remains to be clarified how PDIP38 affects, if it does, mtDNA metabolism through interactions with those single-strand-DNA binding proteins.

Here we show that PDIP38 is located exclusively in the mitochondrial matrix. Little is known about the physiological function of PDIP38, much less its role in mitochondria. We did not always find PDIP38 by mass spectrometry or immunoblotting in mtDNA nucleoids prepared by immunoprecipitation with anti-TFAM antibodies (results not shown), while TFAM was always immunoprecipitated

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from the solubilized mitochondria with anti-PDIP38 antibodies (Fig. 5), suggesting that PDIP38 is possibly a very minor component of the nucleoid, and/or associates unstably with the nucleoid.

In conclusion, PDIP38 may be a minor and/or unstable component of the nucleoids/mitochromosomes, and could be involved in the regulation of mtDNA metabolism.

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