Phosphatidic Acid (PA)-preferring Phospholipase A₁ Regulates Mitochondrial Dynamics*

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Background: Phosphatidic acid (PA) is involved in membrane dynamics.

Results: PA-preferring phospholipase A₁ (PA-PLA₁) affects mitochondrial morphology in an activity-dependent manner. Gene disruption of PA-PLA₁ in mice causes sperm malformation due to mitochondrial organization defects.

Conclusion: PA-PLA₁ regulates mitochondrial dynamics.

Significance: We demonstrate an in vivo function of PA-PLA₁ and suggest a possible mechanism of PA regulation of the mitochondrial membrane.

Recent studies have suggested that phosphatidic acid (PA), a cone-shaped phospholipid that can generate negative curvature of lipid membranes, participates in mitochondrial fusion. However, precise mechanisms underlying the production and consumption of PA on the mitochondrial surface are not fully understood. Recent studies have indicated that PA is involved in a variety of physiological processes such as membrane trafficking and cell signaling. PA can be generated from cardiolipin by the action of phospholipase D (PLD), the phosphorylation of diacylglycerol by diacylglycerol kinase, and the acylation of lysophosphatidic acid (LPA) by lysophosphatidic acid acyltransferase (1, 2). PA can be metabolized to LPA by phospholipases A (PLA₁ and PLA₂) (3) and to diacylglycerol by PA phosphatases such as Lipin 1 (4). Moreover, PA acyl chains may undergo a cycle of deacylation and acylation by means of PLA₁/PLA₂ and lysophosphatidic acid acyltransferases, respectively, in the Land cycle (3, 5).

Mitochondria are dynamic organelles, and their morphology is regulated by the balance between fusion and fission. These processes are mediated by GTPases: fusion of the outer and inner membranes is mediated by Mitofusin (Mfn) and Opal, respectively, and fission is mediated by Drp1 (6). In mitochondria, PA can be generated from cardiolipin by the action of mitochondrial PLD (MitoPLD), a PLD family member found on the mitochondrial surface (7). Recent studies demonstrated that MitoPLD and Lipin 1 phosphatase reciprocally regulate the dynamics of mitochondria. It has been proposed that production of PA by MitoPLD facilitates Mfn-mediated fusion, whereas the consumption of PA and the production of diacylglycerol by Lipin 1 promote mitochondrial fission (8–10).

Intracellular PLA₁ (iPLA₁) proteins constitute a relatively recently discovered lipid-metabolizing enzyme family (11). Mammals have three iPLA₁ family members (phosphatidic acid-preferring phospholipase A₁ (PA-PLA₁)/DDHD1 (12), lysophosphatidic acid: mGPx4, mitochondrial glutathione peroxidase 4; MEF, mouse embryonic fibroblast; Mfn, Mitofusin; MitoPLD, mitochondrial PLD; PA-PLA₁, phosphatidic acid-preferring phospholipase A₁; PLD, phospholipase D; CMXRos, chloromethyl-X-rosamine; PABD, PA binding domain; EGFP, enhanced GFP.
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KIAA0725p/DDHD2 (13), and p125/Sec23IP (14)). PA-PLA<sub>1</sub>, which is the iPLA<sub>1</sub> first identified by Glomset and co-worker (15), preferentially hydrolyzes PA. PA-PLA<sub>1</sub> is highly expressed in brain and testis, especially in mature but not in newborn testis, raising the possibility that PA-PLA<sub>1</sub> is involved in spermatogenesis or sperm function (12, 15). But so far, the physiological role of PA-PLA<sub>1</sub> has not been elucidated. Recent human genetic approaches revealed that mutations in the PA-PLA<sub>1</sub> gene cause hereditary spastic paraplegia (16), an inherited neurodegenerative disorder characterized by slowly progressive lower limb spasticity and weakness (17). Tesson et al. (16) indicated a possible link between lipid metabolism in mitochondria and the appearance of hereditary spastic paraplegia symptoms.

In this study, we showed that PA-PLA<sub>1</sub> regulates mitochondrial dynamics. Ablation of the PA-PLA<sub>1</sub> gene caused deficits in the organization of mitochondria during spermiogenesis, leading to sperm malformation and male subfertility.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The mammalian expression plasmid for FLAG-tagged human PA-PLA<sub>1</sub> was described previously (18). Ser-537 in human PA-PLA<sub>1</sub> corresponds to the active site residue, Ser-540, in bovine PA-PLA<sub>1</sub> (12). The human PA-PLA<sub>1</sub> S37A mutant in which Ser-537 was replaced by Ala was constructed by site-directed mutagenesis. To express FLAG-tagged human PA-PLA<sub>1</sub> and PA-PLA<sub>1</sub> S37A in PA-PLA<sub>1</sub> siRNA-transfected cells, the siRNA targeting sequences were changed by PCR-based site-direct mutagenesis as follows: AAGAGTTGCCTAGATGAAACGAT to AGGAACTTCCGGACAGAT (letters underlined indicate nucleotides changed) for the PA-PLA<sub>1</sub> siRNA5 site. The expression plasmids for the wild-type or mutant (H156N) MitoPLD, Raf1-PA binding domain (PABD)-EGFP, and Raf1-PABD mutant-EGFP were described previously (9). cDNAs encoding myc-tagged wild-type and mutant (H156N) MitoPLD were amplified by PCR and inserted into the pcDNA3 vector.

**RNA Interference**—The siRNA targeting sequences used for HeLa cells were as follows: Lamin A/C siRNA, CTGGACTTCCAGAAGACCA; PA-PLA<sub>1</sub> siRNA2, AAGCCACATTAGAATCCAG; PA-PLA<sub>1</sub> siRNA5, AAGAGTTGCCTATGAAACGAT; KIAA0725p siRNA2, GAAAGAAGATACTGAACCA; KIAA0725p siRNA5, GCGGATTGACTACGTGCTA. PA-PLA<sub>1</sub> was expressed in Sf9 cells and purified using glutathione beads. The purified protein was injected into BALB/c mice, and hybridoma cells producing antibodies against PA-PLA<sub>1</sub> were obtained according to the standard protocol. Culture supernatants were used for the immunostaining of testis sections. Polyclonal antibodies against α-tubulin and FLAG were purchased from Abcam and Sigma-Aldrich, respectively. A polyclonal antibody against SEPT4 was described previously (19). Monoclonal antibodies against cytochrome c and Tom20 were obtained from BD Transduction Laboratories. Monoclonal antibodies against Drp1, Mfn1, and Mfn2 were obtained from BD Transduction Laboratories, Abnova, and Abcam, respectively. FITC-conjugated goat anti-mouse and Texas Red-conjugated goat anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories. Alexa Fluor 350-conjugated goat anti-rabbit and anti-mouse antibodies, Alexa Fluor 488-conjugated goat anti-mouse antibody, MitoTracker Red CMXRos, and MitoTracker Deep Red FM were purchased from Invitrogen. Hoechst 33342 and protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) were obtained from Thermo Scientific and Wako Chemicals, respectively.

**Lipid Analysis by Mass Spectrometry**—Cells were collected by gently scraping with a rubber policeman. Subcellular fractionation was performed essentially as described previously (20), and the mitochondrial fractions were used for the lipid analysis. Protein concentration was determined by the bicinchoninic acid method. Total lipids were extracted by the method of Bligh and Dyer (29). PA and LPA were separated from total lipids using a DEAE-cellulose column. LC-electrospray ionization-MS/MS analysis was performed using a TSQ Vantage (Thermo Fisher Scientific, Waltham, MA) with an UltiMate 3000 LC system (Thermo Fisher Scientific) equipped with an HTPC PAL autosampler (CTC Analytics, Zwingen, Switzerland). This MS system was controlled by Xcalibur software. PA and LPA were measured by selected reaction monitoring in the negative ion mode. The amounts of PA and LPA were determined from the ratio of the peak area of each PA and LPA species to that of internal standards (14:0/14:0 PA and 17:0 LPA).

**Generation of PA-PLA<sub>1</sub> Knock-out Mice**—A targeting vector (Clone PG500049_B_D06) was obtained from the Knock-out Mouse Project (KOMP) Repository at the University of California Davis. The vector was electrooporated into embryonic stem cells (TT2 cells). Two targeted cell lines were injected into ICR eight-cell stage embryos, and chimeric mice were obtained by the conventional method. All animal procedures and experiments were approved by the Animal Care Committee of Tokyo University of Pharmacy and Life Sciences and conducted according to the guidelines of the committee.
Histology—Mouse testes were fixed with 3.7% formaldehyde in PBS, dehydrated, and then embedded in paraffin. Paraffin sections (6 μm) were stained with hematoxylin and eosin (H&E) according to the standard protocol. For immunohistochemistry, testes were fixed with 4% paraformaldehyde in PBS and frozen in O.C.T compound (Tissue-Tek, Sakura, Japan). Cryosections (10 μm) were prepared and stained as described previously (21).

Preparation of Sperm—Cauda epididymis, caput epididymis, and testis were dissected in human tubal fluid medium and incubated with 5% CO₂ at 37 °C for 1 h. Released sperm were washed with PBS and spread on poly-L-lysine-coated coverslips. Then the sperm were fixed with 4% paraformaldehyde for 20 min and used for light microscopy and immunofluorescence analysis. For the motility assay, released sperm were observed under an Olympus IX70 microscope, and images were recorded using an Olympus DP70 charge-coupled device camera.

Immunofluorescence and Microscopic Analyses—Immunofluorescence images were analyzed as described previously (22, 23). An Olympus FluoView 1000 laser scanning microscope was used for confocal microscopy. For electron microscopic analysis, cauda epididymis was sequentially fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and with 1% OsO₄ in the buffer. Electron microscopic analysis was performed as described previously (21).

Preparation of MEFs and Cell Culture—MEFs were isolated from E13.5 embryos by trypsinization and cultured in Dulbecco’s modified Eagle’s medium supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum. Two days after isolation, the cells were immortalized with SV40 large T antigen using a retrovirus expression system (24). The immortalized cells were cultured in medium containing 2 μg/ml puromycin.

RESULTS

PA-PLA₁ Is Involved in Mitochondrial Dynamics—PA-PLA₁ is predominantly expressed in mature testis and brain but also at low levels in other tissues including HeLa cells (Ref. 12 and Fig. 1A). To explore the function of PA-PLA₁ in mitochondrial dynamics, PA-PLA₁ with a FLAG tag was overexpressed in HeLa cells, and then mitochondrial morphology was examined by immunofluorescence microscopy. In control cells, mitochondria exhibited long tubular structures (Fig. 1B, top row). Upon overexpression of PA-PLA₁, the tubular structures became fragmented (middle row). Of note is that this morphological change is activity-dependent. Overexpression of the S537A mutant of human PA-PLA₁, which is equivalent to the S540A mutant of bovine PA-PLA₁ that lacks phospholipase A₁ activity (12), had no effect on mitochondrial morphology (bottom row).

Next, HeLa cells were treated with siRNA targeting PA-PLA₁, and then the morphology of mitochondria was examined. Western blotting verified the depletion of PA-PLA₁ (Fig. 1A, lower panel). In cells transfected with PA-PLA₁ siRNA2, elongation of the mitochondrial tubules was observed (Fig. 2A, middle row). In some cells, interconnected mitochondrial aggregates were detected in the perinuclear region (Fig. 2A, middle row, right column). A similar elongation and aggregation phenotype was observed for cells transfected with PA-
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A

B

C

D

% of cells

0 20 40 60 80 100

Lamin A/C spl #2 spl #5

- Elongated and aggregated
- Intermediate
- Fragmented

Length of mitochondria (μm)

0 2 4 6 8 10 12 14

Lamin A/C, PA-PLA₁, #2, #5

Cytochrome c FLAG Merge

Mock cytochrome c FLAG-PA-PLA₁R WT Merge

PA-PLA₁ #5 siRNA cytochrome c FLAG-PA-PLA₁R S537A Merge

% of cells

0 20 40 60 80 100

Mock, PA-PLA₁, #5 siRNA

FLAG + – – –

FLAG-PA-PLA₁R WT – – + –

FLAG-PA-PLA₁R S537A – – – +
PLA₁ siRNA5 (bottom row). Upon knockdown of PA-PLA₁, the percentage of cells with elongated or aggregated mitochondria was increased from about 10 to about 70% (Fig. 2A, bottom graph). The length of mitochondria in peripheral regions was increased 2.4-fold in cells transfected with PA-PLA₁ siRNA (Fig. 2B). Mitochondrial elongation observed upon PA-PLA₁ knockdown was significantly reversed by expression of siRNA-resistant PA-PLA₁ but not siRNA-resistant S537A mutant (Fig. 2C).

Mitochondrial elongation, interconnection, and aggregation can occur when mitochondrial fusion is promoted or fission is inhibited (25–27). To further verify the effects of the depletion of PA-PLA₁, HeLa cells were treated with CCCP. It has been reported that CCCP causes mitochondrial fragmentation in a Drp1-dependent manner (25). When PA-PLA₁ siRNA2- or siRNA5-transfected cells were treated with CCCP, much less fragmentation of mitochondria was detected (Fig. 2D, middle and bottom rows) compared with control (Lamin A/C siRNA-treated) cells (top row). These results indicate that the overexpression and depletion of PA-PLA₁ cause the fragmentation and elongation of mitochondria, respectively. Note that these effects depend on PA-PLA₁ activity.

PA-PLA₁ Counteracts the Action of MitoPLD on Mitochondria—MitoPLD is a mitochondrial surface PLD that produces PA via hydrolysis of cardiolipin (8). Overexpression of MitoPLD results in mitochondrial aggregation caused by the promotion of fusion events, whereas its siRNA-mediated knockdown results in mitochondrial fragmentation (8). Because PA-PLA₁ is a cytosolic protein and shows phospholipase A₁ activity against PA in vitro (12, 15, 23), it is plausible that PA-PLA₁ hydrolyzes mitochondrial surface PA, thereby regulating mitochondrial morphology. To test this idea, we first analyzed the effects of coexpression of MitoPLD and PA-PLA₁ on mitochondrial morphology (Fig. 3A). As reported previously (8), mitochondria became aggregated in the perinuclear region of MitoPLD-overexpressing cells (Fig. 3A, top row). When PA-PLA₁ was coexpressed with MitoPLD, the mitochondrial aggregation was prevented, and mitochondria spread throughout the cell (middle row). The expression of the S537A mutant had no effect (bottom row). Quantitative data (Fig. 3A, graph) support this viewpoint. Western blotting showed that the expression level of MitoPLD was not affected by coexpression with PA-PLA₁ or the S537A mutant (data not shown).

We next examined the effect of the expression of PA-PLA₁ on mitochondrial surface PA. Raf1-PABD-EGFP is a fluorescent sensor for PA (28) and has been successfully used to detect mitochondrial surface PA generated by MitoPLD (9). When expressed alone, Raf1-PABD-EGFP was localized in the cytosol in many cells (Fig. 3B, top row) but targeted to mitochondria in 28% of Raf1-PABD-EGFP-expressing cells (Fig. 3B, graph). The association of Raf1-PABD-EGFP with mitochondria may be in part due to the hydrophobic nature of Raf1-PABD. Upon coexpression with MitoPLD, Raf1-PABD-EGFP was redistributed from the cytosol to mitochondria (Fig. 3B, third row), and the percentage of cells with Raf1-PABD-EGFP on mitochondria increased to 53%. The binding of Raf1-PABD-EGFP to mitochondria did not significantly increase when a phospholipase D activity-defective mutant (MitoPLD H156N) was expressed (fourth row) or when a Raf1-PABD mutant that lacks PA binding activity (9) was coexpressed with MitoPLD (fifth row). These results demonstrate the specificity of this fluorescent probe. When PA-PLA₁ was coexpressed with Mito-PLD, the targeting of Raf1-PABD-EGFP to mitochondria decreased to the background level (second row from bottom). In contrast, the PA-PLA₁, S537A mutant did not affect the targeting of Raf1-PABD-EGFP to mitochondria (bottom row). The targeting of MitoPLD to mitochondria occurred regardless of whether the expressed PA-PLA₁ construct was the wild type (Fig. 3B, second column from left, second from the bottom row) or the mutant (bottom row), ruling out the possibility that the expression of PA-PLA₁ prevents the attachment of MitoPLD to mitochondria. The expression of PA-PLA₁ alone did not affect the localization of Raf1-PABD-EGFP (second row). Western blotting showed that the expression levels of MitoPLD and Raf1-PABD-EGFP were not affected by coexpression of PA-PLA₁ or the S537A mutant (data not shown). These results suggest that PA-PLA₁ cleaved PA produced by MitoPLD on the mitochondrial surface. Next, we measured mitochondrial PA and LPA by mass spectrometry. Mitochondrial fractions were prepared from control cells, MitoPLD-overexpressing cells, and PA-PLA₁-overexpressing cells. LC-electrospray ionization-MS/MS analysis was performed as described under “Experimental Procedures.” As shown in Fig. 3C, no apparent differences in the content and composition of PA and LPA were observed among the mitochondrial fractions prepared from control, MitoPLD-, and PA-PLA₁-overexpressing cells. These results indicate that the total PA content of mitochondria was not drastically changed upon the overexpression of either MitoPLD or PA-PLA₁. Mitochondrial fission is regulated by Drp1, and the fusion is regulated by Mfn1/2 (6). To examine whether or not
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A

B

C

PA

LPA

Graph A: Bar graph showing the percentage of cells with aggregated mitochondria.

Graph B: Graph showing the percentage of cells.

Graph C: Graph showing the relative intensity of PA and LPA.
PA-PLA₁ directly affects the distribution of these GTPases, PA-PLA₁ was knocked down. As shown in Fig. 4A, treatment of cells with PA-PLA₁ siRNA2 (middle panel) or PA-PLA₁ siRNA5 (bottom panel) did not affect the distribution of Drp1. The enlarged images show that Drp1 was localized in constricted regions of elongated mitochondria (middle and bottom panels, insets). The PA-PLA₁ depletion also did not affect the distribution of Mfn1 (Fig. 4B) or Mfn2 (Fig. 4C). Likewise, overexpression of PA-PLA₁ caused little change in the distributions of these fission and fusion GTPases on mitochondria (data not shown).

Male PA-PLA₁⁻/⁻ Mice Are Subfertile—To elucidate the in vivo function of PA-PLA₁, PA-PLA₁⁻/⁻/geo mice were generated (Fig. 5). The targeting vector was designed to contain exon 8 flanked by two loxP sites. It also contained a splicing acceptor and a geo marker (a fusion between the β-galactosidase and neomycin resistance genes) between exons 7 and 8 of the mouse PA-PLA₁ gene (Fig. 5A). Chimeric mice were obtained by the conventional method, and the male chimeric mice were mated with female wild-type C57BL/6J mice to obtain PA-PLA₁⁺/geo offspring. Southern blotting verified insertion of the geo marker (Fig. 5B). Homozygous PA-PLA₁⁺/geo mice were obtained by intercrossing heterozygous mice. PA-PLA₁⁺/geo/+geo mice showed no significant defects. A slight reduction in fertility was observed in males, but it was not significant (Fig. 5E).

**FIGURE 3.** The ectopic expression of PA-PLA₁ counteracts that of MitoPLD. A, HeLa cells were double transfected with the following plasmid/construct combinations: MitoPLD-myc and FLAG (top row), MitoPLD-myc and FLAG-PA-PLA₁ (middle row), and MitoPLD-myc and FLAG-PA-PLA₁ S537A mutant (bottom row). At 24 h after transfection, the cells were stained with MitoTracker Red CMXRos and antibodies against FLAG and myc. Representative images are shown. Scale bar, 10 μm. The graph shows the percentages of cells exhibiting perinuclear aggregation of mitochondria. At least 100 cells were evaluated for each sample. Data represent the means ± S.D. for three independent experiments. *, p < 0.05, Student’s t test. B, HeLa cells were triple transfected with the following plasmid/construct combinations: Raf1-PABD-EGFP, pcDNA3, and FLAG (top row); Raf1-PABD-EGFP, pcDNA3, and FLAG-PA-PLA₁ (second row); Raf1-PABD-EGFP, MitoPLD-myc, and FLAG-PA-PLA₁ (third row); Raf1-PABD-EGFP, MitoPLD-myc, and FLAG-PA-PLA₁ S537A mutant (fourth row); Raf1-PABD-EGFP, MitoPLD-myc, and FLAG-PA-PLA₁ S537A mutant (mut)-EGFP (fifth row); Raf1-PABD-EGFP, MitoPLD-myc, and FLAG-PA-PLA₁ S537A mutant (bottom row). At 24 h after transfection, the cells were stained with antibodies against FLAG and myc. Representative images are shown. Scale bar, 10 μm. Cells with Raf1-PABD-EGFP on mitochondria were counted, and the quantitative data are shown. At least 100 cells were evaluated for each sample. Data represent the means ± S.D. for three independent experiments. ***, p < 0.01, Student’s t test. C, quantification of PA and LPA contents by mass spectrometry. HeLa cells were double transfected with the empty FLAG plasmid and pcDNA3, the empty FLAG plasmid and the plasmid encoding MitoPLD-myc, or the plasmid encoding FLAG-PA-PLA₁, and FLAG-PA-PLA₁ S537A mutant (bottom row). At 24 h after transfection, the cells were collected, and the quantitative data are shown. Error bars represent S.D.

**FIGURE 4.** Distributions of Drp1 and Mfn1/2 are not affected by depletion of PA-PLA₁. HeLa cells were treated with the indicated siRNAs for 72 h and then double stained with an antibody against Drp1 (A), Mfn1 (B), or Mfn2 (C) and MitoTracker Red CMXRos. Insets are enlarged images of the boxed areas. Scale bar, 10 μm.
We noticed that PA-PLA1geo/geo mice express a tiny amount of PA-PLA1 protein (Fig. 5D). To completely delete the PA-PLA1 gene, PA-PLA1+/geo mice were crossed with transgenic mice expressing Cre recombinase. Heterozygous mice (PA-PLA1−/−) were intercrossed to obtain PA-PLA1−/− mice. Southern blotting verified the loss of exon 8 (Fig. 5C). Western blotting demonstrated a complete loss of PA-PLA1 protein expression (Fig. 5D). PA-PLA1−/− mice were apparently normal. However, PA-PLA1−/− male mice were found to be subfertile (Fig. 5E). The number of sperm (Fig. 5F, left graph), testis weight (Fig. 5F, right graph), and germ cell arrangement in seminiferous tubules (Fig. 5G) were almost normal in PA-PLA1−/− mice.

PA-PLA1 Is Required for Sperm Tail Formation—We then analyzed spermatozoa collected from the cauda epididymis by...
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**FIGURE 6.** Spermatozoa from PA-PLA<sub>1</sub><sup>−/−</sup> mice are bent at the junction of the middle and principal pieces. A, differential interference contrast images of spermatozoa derived from the cauda epididymis of PA-PLA<sub>1</sub>+/+ and PA-PLA<sub>1</sub><sup>−/−</sup> mice. The lower row shows enlarged images of the boxed areas in the upper row. Scale bar, 20 μm. B, spermatozoa from the cauda epididymis of PA-PLA<sub>1</sub>+/+ and PA-PLA<sub>1</sub><sup>−/−</sup> mice were triple stained with Hoechst 33342 (blue), MitoTracker Red CMXRos (red), and anti-α-tubulin antibody (green). The lower row shows enlarged images of the boxed areas in the upper row. Scale bar, 20 μm. C, motility of spermatozoa derived from the cauda epididymis was recorded for 10 s. At least 100 spermatozoa were assessed. The data are the averages for two independent experiments. D, spermatozoa collected from the cauda epididymis of PA-PLA<sub>1</sub>+/+ and PA-PLA<sub>1</sub><sup>−/−</sup> mice were triple stained with Hoechst 33342 (blue), anti-SEPT4 antibody (green), and mitochondrial membrane potential-sensitive dye MitoTracker Deep Red FM (red). Arrows indicate the sites where the MitoTracker staining was absent. Scale bar, 20 μm.

Differential interference contrast microscopy. Strikingly, spermatozoa from PA-PLA<sub>1</sub><sup>−/−</sup> mice were sharply bent between the middle and principal pieces, exhibiting a hairpin-like structure (Fig. 6A). Microtubules were disrupted at that position (Fig. 6B), and about 90% of the spermatozoa from PA-PLA<sub>1</sub><sup>−/−</sup> mice exhibited impaired movement (Fig. 6C). It has been reported that a defect in the annulus, a fibrous ring structure connecting the middle and principal pieces of the mammalian sperm flagellum (30), causes the structure to be bent between the middle and principal pieces (19). Staining with an antibody against SEPT4, a main structural component of the annulus (19), revealed that spermatozoa from PA-PLA<sub>1</sub><sup>−/−</sup> mice possessed an annulus that was not attached to the mitochondrial sheath, whereas that in control mice was attached. A small gap structure, which was not stained with MitoTracker, was present between the mitochondrial sheath and the annulus in spermatozoa from PA-PLA<sub>1</sub><sup>−/−</sup> mice (Fig. 6D, arrows).

**FIGURE 5.** Subfertility of male PA-PLA<sub>1</sub><sup>−/−</sup> mice. A, schematic representation of the wild-type (+), targeted (geo), and knock-out (−) alleles. The lipase consensus sequence SHSLG is encoded in exon 10. Cre-mediated deletion of exon 8 is supposed to cause a frameshift. Exons of the PA-PLA<sub>1</sub> gene and loxP sequences are indicated by black boxes and closed triangles, respectively. FRT, Flp recombination target; SA, splice acceptor sequence of mouse engrailed 2; LacZ, β-galactosidase gene; Neo', neomycin resistance gene; pA, SV40 polyadenylation signal. B, Southern blot analysis of EcoRI-digested genomic DNA derived from the wild-type allele (+/+) and heterozygous targeted allele (+/geo). The S′ probe in A recognized 13- and 16-kb fragments of the wild-type and targeted alleles, respectively. g denotes geo. C, Southern blot analysis of EcoRV-digested genomic DNA from the wild-type (+/+), heterozygous targeted (+/geo), homozygous targeted (geo/geo), heterozygous (+/−), and knock-out (−/−) alleles. The Neo probe in A recognized 3.3- and 20-kb fragments of the targeted and knock-out alleles, respectively. D, testis lysates (100 μg) were analyzed by Western blotting with a monoclonal antibody against PA-PLA<sub>1</sub>, and an anti-α-tubulin antibody. The bottom panel shows a longer exposure image. E, average litter size and pregnancy rate of PA-PLA<sub>1</sub><sup>−/−</sup> mice. The numbers of matings were 55, 66, and 24 for male +/+ , g/g, and −/−, respectively. ♀, female. The average litter sizes were calculated as the number of pups per number of matings. Error bars represent the S.E. for each set of crossing experiments. F, the average number of spermatozoa collected from the cauda epididymis. Data are means ± S.E. (+/+, n = 11; −/−, n = 10) (left graph). Testis weight was normalized as to body weight. Data are means ± S.E. (+/+, n = 9; −/−, n = 9) (right graph). G, H&E staining of seminiferous tubules of testes from PA-PLA<sub>1</sub><sup>+/+</sup> and PA-PLA<sub>1</sub><sup>−/−</sup> mice. Scale bar, 40 μm.
Results showed that PA-PLA\textsubscript{1} protein was expressed in round spermatids with a monoclonal antibody against PA-PLA\textsubscript{1} during spermiogenesis. A total protein lysates (50 \textmu g) of testis and cauda epididymis were analyzed by Western blotting (Fig. 8A). Testis sections from PA-PLA\textsubscript{1}\textsuperscript{+/+} and PA-PLA\textsubscript{1}\textsuperscript{−/−} mice were double stained with a monoclonal antibody against PA-PLA\textsubscript{1} (green) and Hoechst 33342 (blue). Staining patterns of stages I–VI, VII–VIII, and IX–XII of the seminiferous epithelium cycle are shown. The intense staining between seminiferous epithelium cycle was observed in PA-PLA\textsubscript{1}−/− cross-sections (third row), indicating the specificity of the antibody. Scale bars, 40 \textmu m.

**FIGURE 7.** Stage-specific expression of PA-PLA\textsubscript{1} during spermatogenesis. A, total protein lysates of testis and cauda epididymis were analyzed by Western blotting. B, testis sections from PA-PLA\textsubscript{1}−/− mice were double stained with a monoclonal antibody against PA-PLA\textsubscript{1} (green) and Hoechst 33342 (blue). Staining patterns of stages I–VI, VII–VIII, and IX–XII of the seminiferous epithelium cycle are shown. The intense staining between seminiferous tubules is due to nonspecific binding of secondary antibody. No staining was observed in PA-PLA\textsubscript{1}−/− cross-sections (third row), indicating the specificity of the antibody. Scale bars, 40 \textmu m.

**PA-PLA\textsubscript{1} Is Involved in the Organization of Mitochondria during Spermiogenesis**—To elucidate how loss of PA-PLA\textsubscript{1} causes the abnormal structure comprising a bent sperm flagellum, we first examined at which stage PA-PLA\textsubscript{1} is expressed during spermatogenesis. The PA-PLA\textsubscript{1} protein was detected in testicular cells but not in the cauda epididymis containing mature sperm (Fig. 7A). The expression of PA-PLA\textsubscript{1} was further examined by immunostaining of cross-sections of seminiferous tubules with a monoclonal antibody against PA-PLA\textsubscript{1}. Results showed that PA-PLA\textsubscript{1} protein was expressed in round and elongating spermatids but not in spermatocytes or spermatogonia (Fig. 7B, top row). Relatively strong staining was observed at stages IX–XII of the cycle of seminiferous epithelium (31), which contained elongating spermatids (steps 9–12).

Spermatozoa formed in the testis enter the caput epididymis and then migrate to the cauda region where they are stored. To clarify when the hairpin-like structure is created, we compared the shapes of spermatozoa collected from the testis, caput epididymis, and cauda epididymis of PA-PLA\textsubscript{1}−/− mice. As shown in Fig. 8A, the ratio of spermatozoa with the hairpin-like structure increased as sperm migrated from the testis to the cauda region. The ratio of spermatozoa with an abnormal microtubule structure increased similarly (Fig. 8B). Most of the spermatozoa from PA-PLA\textsubscript{1}−/− testis and caput epididymis were found to have a straight junction between the middle and principal pieces (Fig. 8A). In these spermatozoa, a narrow segment adjacent to the mitochondrial sheath was observed, and the mitochondrial sheath and principal piece were separated by this short region (Fig. 8A, enlarged image, arrowheads). This narrow segment corresponds to the gap structure between the mitochondrial sheath and the annulus (Fig. 6D, arrows). As shown in Fig. 8C, the lengths of the mitochondrial sheaths of spermatozoa prepared from testis, caput epididymis, and cauda epididymis of PA-PLA\textsubscript{1}−/− mice were almost identical but were shorter than those in PA-PLA\textsubscript{1}+/+ mice. These results suggest that the shortened mitochondrial sheath is the cause of the gap structure between the middle and principal pieces.

The sperm defects found in PA-PLA\textsubscript{1}−/− mice are quite similar to those recently reported in mitochondrial glutathione peroxidase 4 (mGPx4) knock-out mice (32, 33). mGPx4 is a structural component of the sperm mitochondria capsule. Spermatozoa of mGPx4 knock-out mice have a truncated mitochondrial sheath, and their mitochondrial structure is irregular and swollen. To examine the structure of mitochondria in spermatozoa from PA-PLA\textsubscript{1}−/− mice, electron microscopy was performed. Results showed that mitochondria in spermatozoa from PA-PLA\textsubscript{1}−/− mice are irregular and aligned in a disorganized manner (Fig. 8D). Overall, it is most likely that spermatozoa produced in PA-PLA\textsubscript{1}−/− mice testes possess a disorganized mitochondrial sheath. Because of the absence of rigid connection between the middle and principal pieces, PA-PLA\textsubscript{1}−/− spermatozoa may become bent during their migration to the cauda epididymis and/or when their flagella start moving in the cauda epididymis. These results suggest the importance of PA-PLA\textsubscript{1} in the mitochondrial organization during spermiogenesis.

**KIAA0725p Is Involved in the Mitochondrial Dynamics in MEFs**—We examined whether mitochondrial morphology is also affected in MEFs derived from PA-PLA\textsubscript{1}−/− mice. In contrast to spermatids, no apparent morphological change in mitochondria was observed (Fig. 9).

KIAA0725p is a member of the iPLA\textsubscript{1} family and, like PA-PLA\textsubscript{1}, shows phospholipase A\textsubscript{2} activity against PA in vitro (13, 23). KIAA0725p is expressed broadly in many tissues (13). To test the possibility that KIAA0725p is also involved in mitochondrial dynamics, KIAA0725p was knocked down in HeLa cells (Fig. 10A). Differing from the case of PA-PLA\textsubscript{1}, siRNA-mediated knockdown of KIAA0725p did not cause the elongation or aggregation of mitochondria. We showed previously that the subcellular localization of KIAA0725p is different...
among cell lines (22). In HeLa cells, KIAA0725p is localized to the Golgi and the cytosol, whereas it is predominantly localized to the cytosol in MEFs. We next examined the effects of KIAA0725p depletion in MEFs. MEFs were treated with siRNAs targeting PA-PLA₁ or KIAA0725p, and then the morphology of mitochondria was examined (Fig. 10B). siRNA-
mediated knockdown of PA-PLA₁ did not cause significant changes in mitochondrial morphology in MEFs. Instead, elongation of mitochondrial tubules was observed in MEFs transfected with KIAA0725p siRNA1 (Fig. 10B, fourth row) and siRNA5 (bottom row). In some cells, interconnected mitochondrial aggregates were detected in the perinuclear region (Fig. 10B, fourth and bottom rows, right column). These results suggest that KIAA0725p is involved in the regulation of mitochondrial morphology in MEFs.

**DISCUSSION**

In this study, we demonstrated that PA-PLA₁ regulates mitochondrial morphology. A specific physiological role of PA-PLA₁ in mitochondrial function was verified in PA-PLA₁-deficient sperm. It is a little surprising that PA-PLA₁ affects mitochondria. Because PA-PLA₁ is a cytosolic protein (15), it can affect all membranes in contact with the cytosol. We cannot exclude the possibility that other organelle membranes are also affected by PA-PLA₁, but our results suggest that mitochondria are one of the critical targets of PA-PLA₁. The phenotype of PA-PLA₁⁻/⁻ mice is quite similar to that of mGPx4⁻/⁻ mice (32, 33), supporting the idea that PA-PLA₁ affects mitochondria. Interestingly, the YOR022c protein, the sole iPLA₁ in Saccharomyces cerevisiae, was reported to be localized in mitochondria (34). A recent genome-wide interaction study showed that YOR022c genetically interacts with several genes implicated in mitochondrial dynamics and mitochondrial lipid metabolism. Examples of the genes include UGO1, an outer membrane component implicated in mitochondrial membrane fusion, and UPS1, a phosphatidic acid transfer protein in mitochondria (35).

PA-PLA₁ exhibits PLA₁ activity toward PA (12, 15, 23). The overexpression and depletion of PA-PLA₁ cause the fragmentation and elongation of mitochondria, respectively. MitoPLD has been postulated to facilitate Mfn-mediated mitochondrial fusion by producing PA from cardiolipin (8). Overexpression of PA-PLA₁ counteracts that of MitoPLD in terms of mitochondrial dynamics. These results encouraged us to propose a working model that PA-PLA₁ hydrolyzes mitochondrial surface PA and thereby regulates mitochondrial morphology. Our results using Raf1-PABD as a PA sensor support this model. However, lipid analysis by mass spectrometry failed to show significant changes in the PA content of mitochondria upon overexpression of MitoPLD or PA-PLA₁. It is possible that a small fraction of the total mitochondrial lipids might be metabolized by MitoPLD and PA-PLA₁. More sensitive and direct measurement of mitochondrial surface PA is necessary to further verify the model.

Drp1, which is essential for mitochondrial fission (6), was normally targeted to constricted regions of elongated mitochondria in PA-PLA₁-knocked down cells (Fig. 4A). Moreover, knockdown of PA-PLA₁ retarded CCCP-induced mitochondrial fragmentation (Fig. 2C). These results suggest that PA-PLA₁ knockdown does not affect the association of Drp1 with mitochondria but may inhibit the fission of constricted mitochondria. PA and LPA are cone- and inverted cone-shaped lipids that generate negative and positive curvature of membranes, respectively (3). The PA consumption and/or LPA production by PA-PLA₁ at mitochondrial constriction sites might be important for mitochondrial fission. The viewpoint that LPA favors the fission of mitochondria is consistent with our recent study, which demonstrated that CI-976, an inhibitor for lysophospholipid acyltransferases such as lysophosphatidic acid acyltransferase (3), causes mitochondrial fragmentation (22). However, given that mitochondrial morphology is regulated by the balance between fusion and fission, it is also possible that PA-PLA₁ negatively regulates Mfn-mediated fusion rather than stimulates mitochondrial fission. It has been reported that phospholipase D₁ acts on the mitochondrial sur-
In addition, PA can be generated by the phosphorylation of diacylglycerol and the acylation of LPA. It is possible that PA-PLA₁ metabolizes PA produced via several routes. The results of knock-out mice studies suggest a possible functional link between PA-PLA₁ and Mito-PLD in vivo. Mito-PLD ablation causes meiotic arrest during spermatogenesis (9, 38). In MitoPLD-depleted cells, the formation of the nuage, a perinuclear structure involved in piRNA biogenesis/function (39), is impaired (9, 38). Huang et al. (9) have proposed that PA produced on the surface of mitochondria by MitoPLD recruits or activates nuage components. Consistent with this idea, expression of MitoPLD is high in spermatocytes undergoing meiosis and low in round spermatids. Meanwhile, PA-PLA₁ protein is expressed in round and elongating spermatids but not in spermatocytes. Because the nuage acts as intermitochondrial “cement” (39), it may prohibit the reorganization of mitochondria required for subsequent flagellum formation. As spermatogenesis progresses, PA-PLA₁ may metabolize PA and thus adjust the PA level for subsequent mitochondrial reorganization steps in spermatids. MitoPLD might be involved in the formation of piRNA in early stages of spermatogenesis and later in the regulation of mitochondrial morphology. Indeed, loss of MitoPLD affects the mitochondrial morphology in MEFs (9), suggesting that its role is not limited to piRNA generation. Huang et al. (9) have suggested that a PA phosphatase, Lipin 1, may metabolize PA produced by MitoPLD. The relationship between PA-PLA₁ and Lipin 1 in the regulation of mitochondrial dynamics should be clarified in the future.

**FIGURE 10.** KIAA0725p regulates mitochondrial dynamics in MEFs. HeLa cells (A) or MEFs (B) were treated with the indicated siRNAs for 72 h and subsequently stained with an antibody against cytochrome c. Higher magnification views of the boxed areas are shown on the right. Scale bar, 10 µm. B, quantitative data are shown at the bottom. Cells in which most mitochondria were elongated or aggregated were counted. At least 100 cells were analyzed. Data represent the means ± S.D. for three independent experiments. Error bars represent S.D.
align along a flagellum in late elongating spermatids, e.g. step 15 in mice. Prior to this alignment step, pronounced expression of the fusion and fission factors is observed in late round spermatids and early elongating spermatids (40, 41). One report suggested the necessity of intensive homogenization of mitochondria at a certain stage of spermiogenesis (i.e. steps 8–12) (41). It is plausible that PA-PLA₁ might be involved in the regulation of mitochondrial fusion and fission at this stage.

A remaining question concerning iPLA₁ in mammals concerns functional separation or redundancy between PA-PLA₁ and KIAA0725p. KIAA0725p exhibits an enzyme activity similar to that of PA-PLA₁ (13, 23). Recent reports have mentioned mutations of the PA-PLA₁/DDHD1 gene (16) and KIAA0725p/DDHD2 gene (42, 43) in hereditary spastic paraplegia patients, suggesting that the two proteins have similar physiological significance in the nervous system. Mutations in the Lipin 1 gene (44, 45) and rats (46) induce hind limb paralysis, which suggests the importance of PA metabolism in these rodents. Although PA-PLA₁⁻/⁻ mice did not show significant gait disturbance, precise neurological examination will be necessary to determine the effect of PA-PLA₁ gene deletion on the nervous system. Redundant functions between PA-PLA₁ and KIAA0725p may explain the fact that mitochondria in MEFs derived from PA-PLA₁⁻/⁻ mice show a normal morphology (Fig. 9). Consistent with this idea, KIAA0725p knockdown caused mitochondrial elongation in MEFs but not in HEla cells (Fig. 10). KIAA0725p is predominantly localized to the cytosol in MEFs (22). Perhaps, cytosolic KIAA0725p can act on mitochondrial surface lipids in a manner similar to PA-PLA₁. The expression levels and cellular distribution of PA-PLA₁ and KIAA0725p may determine which protein mainly functions in each cellular process.

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