PGAP1 Knock-out Mice Show Otocephaly and Male Infertility*

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A palmitate linked to the inositol in glycosylphosphatidylinositol (GPI) is removed in the endoplasmic reticulum immediately after the conjugation of GPI with proteins in most cells. Previously, we identified PGAP1 (post GPI attachment to proteins 1) as a GPI inositoldeacylase that removes the palmitate from inositol. A defect in PGAP1 caused a delay in the transport of GPI-anchored proteins (GPI-APs) from the endoplasmic reticulum to the cell surface in Chinese hamster ovary cells, although the cell-surface expression of GPI-APs in the steady state was normal. Nevertheless, in most cells, GPI-APs undergo deacylation. To elucidate the biological significance of PGAP1 in vivo, we established PGAP1 knock-out mice. Most PGAP1 knock-out mice showed otocephaly, a developmental defect, and died right after birth. However, some survived with growth retardation. Male knock-out mice showed severely reduced fertility despite the capability of ejaculation. Their spermatozoa were normal in number, motility, and ability to ascend the uterus, but were unable to go into the oviduct. In vitro, PGAP1-deficient spermatozoa showed weak attachment to the zona pellucida and a severely diminished rate of fertilization. Therefore, an extra acyl chain in GPI anchors caused severe deleterious effects to development and sperm function.

Many eukaryotic cell-surface proteins with various functions are anchored to the membrane via glycosylphosphatidylinositol (GPI) (1–3). The structure of GPI is well conserved among eukaryotes. More than 150 GPI-anchored proteins (GPI-APs) with various functions have been identified, including complement system regulatory factors (CD55 and CD59), enzymes (acetylcholinesterase, alkaline phosphatase, and others), adhesion molecules (CD48, neural cell adhesion molecule, and others), and receptors for signal transduction (lipopolysaccharide receptor, urokinase-type plasminogen activator receptor, folate receptor, and others) (3, 4). The GPI anchor is synthesized in the endoplasmic reticulum (ER), consisting of ethanolamine phosphate, three mannoses, glucosamine, and phosphatidylinositol (3, 5, 6), and is transferred to proteins. Phosphatidylinositol glycan A is the enzyme involved in the early step of GPI synthesis, and its disruption in mice causes complete loss of GPI synthesis resulting in embryonic lethality with defective gastrulation and neural development indicating the importance of GPI-APs for normal development (7).

At an early step, the inositol ring of GPI is acylated with a palmitoyl chain by phosphatidylinositol glycan W (8). This inositol acylation is indispensable for the attachment of ethanolamine phosphate to the third mannose of GPI intermediates, and phosphatidylinositol glycan W-defective mutant cells express very low levels of GPI-APs (8). Soon after the attachment of mature GPI to proteins, the inositol is usually decylated in the ER (9). This process is necessary for efficient transport of GPI-APs to the plasma membrane by vesicular trafficking (10). GPI-APs accumulate in specific microdomains in the plasma membrane called lipid rafts, which are enriched in sphingolipids and cholesterol (11–13). GPI-APs are incorporated into lipid rafts in the Golgi and transported to the cell surface (11). Rafts are the portals for endocytosis and the platforms for signal transduction and mediate apical sorting of GPI-APs (12–15). Previously, we established mutant cells based on resistance to bacterial PI-PLC, which cleaves GPI-APs unless palmitoylated on the inositol (16–18), and identified PGAP1 as the inositoldeacylase (10). A clear delay in the glycosylation of GPI-APs was observed, and the accumulation of ER-form GPI-APs was obvious, indicating the transport of GPI-APs from ER to Golgi was delayed; however, cell-surface expression of GPI-APs was not affected (10). On most cells, except human erythrocytes, GPI-APs are decylated (16–18). Why should most GPI-APs on the membrane be decylated? To clarify the physiological role of this decylation, we generated PGAP1 knock-out mice.

EXPERIMENTAL PROCEDURES

Generation of PGAP1-deficient Mice—The targeting vector for deletion of PGAP1 exon 5 contains an expression cassette of the neomycin resistance gene (neo) for positive selection and diphteria toxin for negative selection. The target vector was linearized by digestion with Asc-I and electroporated into mouse D3 embryonic stem cells. Homologous recombinants were selected using G418, PCR, and Southern blotting and were injected into C57BL6 mouse blastocysts. PCR for the first embryonic stem cell screening was performed using primer1 (5′-TAAGCTTgaagAACAACAgAT-3′) and primer2 (5′-cAGagCCAGAATTCATG-3′) with the neo gene as the probe.
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ACTCAT-3') and primer2 (5'-gTTATTCCgTCCCTCgAAATAgg-TTCA-3'). Chimeric male mice were crossed with C57BL/6 J female mice (CLEA Japan, Inc., Tokyo) to establish heterozygous mutant lines. Genotyping was performed with the pair of primer3 (5'-TCCATgGTCgTTTGgTAATCgT-3') and primer4 (5'-TAGCAGAAATACACCTAgGAgCACCC-3') for wild type, generating a 355-bp band, and the pair of primer3 and primer5 (5'-gTTATTAggTCCCTCgAAAggTTCA-3') for knock-out mice, generating a 269-kb band. Homozygous knock-out mice were obtained by crossing heterozygous male with female mice. All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals in Osaka University.

Southern Blotting—Genomic DNA was prepared from the tail, digested with BsPII or NdeI, and separated by agarose gel electrophoresis. The probe B was generated by PCR using the set of primer A (5'-gAGgCggCCAAgAgTAGgggTTAgAggTTgG-3') and primer B (5'-gTTATTAgATgAgTTATCgTlgTTgTgCTCCT-3'), and the probe N was generated by PCR using the set of primer C (5'-ACTgAAACACTTTgTgAATgAggAA-3') and primer D (5'-ATAAggCACTTTgTgATAgCTCaATg-3').

PI-PLC Assay—White blood cells derived from 300 μl of peripheral blood were diluted with 10 μl of Dulbecco’s modified Eagle’s medium containing 1 unit/ml PI-PLC (Molecular Probes) and 0.02 M HEPES buffer (Sigma). Samples were incubated at 37 °C for 30 min, and the cells were subjected to fluorescence-activated cell sorting analysis. The antibodies used were allophycocyanin-conjugated anti-mouse CD4 (RM4–5), fluorescein isothiocyanate-conjugated anti-mouse CD8 (Ly-2), phycoerythrin-conjugated Armenian hamster anti-mouse CD48 (BCM1, HM48–1, IgG1, and lambda3, all from BD Pharmingen). Phycoerythrin-conjugated Armenian hamster anti-mouse T cell receptor β chain (H57–597) and phycoerythrin-conjugated Armenian hamster anti-mouse CD48 (BCM1, HM48–1, IgG1, and lambda3, all from BD Pharmingen). Phycoerythrin-conjugated Armenian hamster anti-mouse T cell receptor β chain (H57–597) and phycoerythrin-conjugated Armenian hamster anti-mouse CD48 (BCM1, HM48–1, IgG1, and lambda3, all from BD Pharmingen).

In Vivo Mating—Each experimental and control mouse was mated with 3 female BDF-1 mice for 8 weeks. The females were allowed to deliver the pups, which were counted at birth. The females that failed to become pregnant when mated with PGAP1−/− mice were later tested for fertility by mating with normal males.

Sperm Migration Assay—Ascent of sperm from the uterus into oviduct was examined as described previously (19). B6DF1 female mice at 8 weeks of age were superovulated by intraperitoneal injection of 5 units of pregnant mare’s serum gonadotropin (Teikoku Zoki Co., Tokyo), followed by injection of 5 units of human chorionic gonadotropin (Teikoku Zoki Co.) 48 h later. Superovulated females were mated with PGAP1 mutant males of each genotype 12 h after human chorionic gonadotropin injection, and the formation of a vaginal plug was observed every 30 min. About 2 h after copulation, oviducts were excised with the connective part of the uterus, fixed in PBS containing 4% paraformaldehyde for 6 h at 4 °C, washed with PBS, and frozen in OCT compound (Sakura Finetechical Co., Tokyo) with liquid nitrogen. Sections were prepared, stained with hematoxylin, and then observed under a microscope.

In Vitro Fertilization—The capacitation was induced in vitro by incubating sperm from PGAP1 homozygous mutant and wild-type littersmates in TYH medium (modified Krebs-Ringer bicarbonate solution containing glucose, sodium pyruvate, bovine serum albumin, and antibiotics) (20). Ovulated egg masses from B6DF1 mice (>2 months old, Japan SLC, Inc., Shizuoka, Japan) were placed in a 200-μl drop of TYH medium. An aliquot of capacitated sperm (2 × 105 sperm/ml) was inseminated. The mixture was incubated for 8 h at 37 °C under 5% CO2 in air (21). Approximately 60–80 eggs for each genotype were examined for fertility by pronuclei formation. For the sperm-zona binding assay, egg masses were treated with bovine testicular hyaluronidase (175 units/ml, Sigma) for 5 min to remove the cumulus cells. Cumulus-free eggs were placed in a 200-μl TYH drop and inseminated. After 30 min of incubation, sperm bound to the zona pellucida of the eggs were observed using an IX-70 microscope (Olympus). We did not differentiate the state of sperm on the zona pellucida, “attaching” and “binding,” so the eggs were observed without washing by pipetting.

Antibodies—Affinity-purified rabbit anti-SPAM1 (PH-20) antibody was a kind gift from Dr. Tadashi Baba (University of Tsukuba, Ibaraki, Japan) (22). Monoclonal antibodies against mouse ADAM2 (9D2.2) and ADAM3 (7C1.2) were purchased from Chemicon International, Inc. Monoclonal antibody against TACE (1D5) was prepared as described (23). An Armenian hamster monoclonal antibody against mouse GPI-DAF (RIKO-4) was a kind gift from Drs. Hidechika Okada and Noriko Okada (Nagoya City University, Nagoya, Japan) (24). A polyclonal antibody against Izumo was prepared as described (25). An antibody against CD59b was a kind gift from Dr. B. Paul Morgan (University of Wales College of Medicine, UK) (26).

Preparation of Protein Extracts—Mice testes were washed with cold PBS, and their covering tunica albuginea were removed in cold PBS. Testicular tubes were further washed in PBS and homogenized in 25 volumes of lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride) using a Micro Multi Mixer (IEDA Trading Corp., Tokyo). The protein concentrations of homogenates of each sample were measured using a BCA™ Protein Assay Kit (Pierce) and adjusted appropriately.

Mouse sperm were collected from the cauda epididymis and vas deferens, washed with 4°C PBS once, and centrifuged at 810 × g for 3 min at 4°C. The pellet was lysed with the same lysis buffer, and the protein concentration was adjusted in the same manner as testes.

Western Blotting—Protein extracts were centrifuged at 15,000 × g for 10 min at 4°C to remove the insoluble protein and debris, and the supernatants were collected. Proteins were separated by SDS-PAGE under reducing conditions (ADAM2, ADAM3, tACE, and Izumo) or non-reducing conditions (CD55, CD59b, SPAM-1, CD52) and transferred electrophoretically to polyvinylidene difluoride membranes. After blocking with TBS-T buffer (20 mM Tris-HCl (pH7.4), 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk overnight, blots were incubated with primary antibodies for 1 h at room temperature. Membranes were
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RESULTS

Generating PGAP1 Knock-out Mice—The targeting construct for generating PGAP1 knock-out mice was designed to delete exon 5 of the PGAP1 gene, which contains a serine-containing catalytic motif (10). Five chimeric male mice were able to sire PGAP1+/− mice. The analyses of PGAP1 knock-out mouse in this report are basically based on the first PGAP1−/− line, and the phenotypes observed in the first line were also examined in other lines derived from different embryonic stem cells. The genotypes of wild-type (PGAP1+/+), heterozygous (PGAP1+/−), and homozygous mutant (PGAP1−/−) mice were identified by PCR and Southern blot analyses of genomic DNA (Fig. 1). Peripheral blood cells of each genotype were tested for PI-PLC resistance. CD48, a GPI-AP, on wild-type but not knock-out TCR-β-positive mononuclear cells was sensitive to PI-PLC (Fig. 2). The same result was observed with B220-positive mononuclear cells (data not shown), indicating that PGAP1 was successfully knocked out in homozygous mutants.

High Frequency of Perinatal Death and Otocephaly of PGAP1 Knock-out Mice—Breeding of PGAP1+/− males and female mice resulted in a smaller number of homozygous mutant mice than expected from Mendelian genetics (PGAP1−/−:PGAP1+/+−PGAP1+/+ = 55(26%):122(57%):36(17%)). Most PGAP1−/− (31 out of 36 pups) died right after delivery or within 24 h of birth. More than half of the dead homozygous mutant mice showed severely disturbed face and jaw shaping, which seemed similar to the phenotype of otocephaly in mammalian species and humans (27–30) (Fig. 3). The severity of otocephaly varied among the knock-out mice from a normal face to a complete lack of mouth and jaw. Even the most affected were alive on delivery but soon became cyanotic and died. PGAP1 knock-out mice with normal faces were active and vigorous after birth, but milk was not observed in their stomachs when they died, even though behaviors of heterozygous mothers (retrieval and licking the pups) were observed. Those who survived the critical 24 h after birth were able to survive like other littermates, and finally we obtained four male and one female adult homozygous knock-out mice. The number of PGAP1−/− mice, even including dead ones, was smaller than that of expected from Mendelian genetics. To examine the possibility of embryonic lethality, we performed cesarean operations on pregnant mice at 18.5 days post coitus. Genotypes of 30 embryos from 3 mice were PGAP1+/+:

![Diagram showing genotyping by PCR and Southern blot analysis](image1)

![Diagram showing restriction sites for BspHI and NdeI](image2)

then washed with TBS-T buffer twice and incubated with the appropriate second antibodies. Detection of bands was performed using a Western Lightning™ kit (PerkinElmer Life Sciences). Signal intensities were determined using the Fujifilm LAS-1000 system (Fuji Photo Film Co., Ltd., Japan). Triton X-114-mediated partitioning of proteins into aqueous and detergent phases was done as described (10).

Statistical Analysis—All the statistical analyses were done with JMP Start Statistics (SAS Institute Inc.).

FIGURE 1. In A, the top diagram is a schematic representation of the normal PGAP1 allele; the middle diagram shows the targeting construct with PGK neomycin (neo) as a positive selectable marker and diphtheria toxin (DT) as a negative selective marker. This construct was designed to delete the PGAP1 gene. The bottom diagram shows the PGAP1 allele mutated by homologous recombination. The exons are represented by blank boxes. PCR primers for homologous recombination screening are represented by the short arrows labeled U and L. B, genotyping by Southern blot analysis of DNA purified from the tails of F2 mice generated from crossing with PGAP1+/− F1 pups. After cutting with the restriction enzymes BspHI (for the upstream part) or NdeI (for the downstream part), DNA was hybridized with non-radioisotope-labeled probes shown as Probe B (for the upstream part) or Probe N (for the downstream part). The bold “B” or “N” in panel A shows restriction sites for BspHI or NdeI. C, genotyping by PCR was performed using three kinds of primers. PGAP1 (−/−) was detected as a single band at 269 bp using the pair of primer3 and primer5, PGAP1 (+/+ ) was detected at 355 bp using the pair of primer3 and primer4, and PGAP1 (+/−) mice express both bands.
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![Graph showing PI-PLC resistance of peripheral blood of each genotype.](image)

**FIGURE 2.** PI-PLC resistance of peripheral blood of each genotype. Peripheral blood of knock-out or wild-type mouse was treated with (+) or without (−) PI-PLC for 30 min at 37 °C, and the surface expression of CD48 was analyzed by fluorescence-activated cell sorting. The dotted line shows the staining obtained with an isotype control for the anti-CD48 antibody.

PGAP1+/−:PGAP1−/− = 5:18.7. Four out of seven homozygous mutant mice showed otocephaly, but all of them were still alive in the amnia, suggesting that pups die immediately after birth. A comparison of sections from pups of each genotype showed that the brains of most homozygous mutants were degenerated, but that other organs looked intact. Most of the PGAP1−/− embryos lacked rostral structures. In PGAP1−/−, the oropharynx and mandible were developed, but the size of the mandible was small. In severely affected ones, no maxillary bone, nasal cavity, nasal sinus, or olfactory bulb was observed. Although the formation of telencephalon was detected in both PGAP1+/+ and PGAP1−/− embryos, the layer structure of PGAP1−/− embryos was immature. The cerebral cortex of PGAP1+/+ embryos consisted of layers arranged parallel to the meningeal surface. The density and number of cells in the most superficial layer of PGAP1−/− mice was lower than that in PGAP1+/+ mice, indicating an immature structure (Fig. 3). 43 embryos at 17.5–18.5 days post coitus derived from another embryonic stem clone had genotypes PGAP1+/−:PGAP1+/−: PGAP1−/− = 13:17:13. Three of the thirteen homozygous mutant mice showed severe otocephaly, two showed smaller faces, but all of the homozygous mutant pups were alive in the amnia.

**PGAP1 Knock-out Mice Show Growth Retardation**—Peripheral blood of each genotype was used in serological tests for liver function, kidney function, and other biochemical tests, but no clear deviation was observed among the genotypes. Blood cell counts and isotype deviation of CD4+/8+ cells in T cells, or B/T cells in lymphocytes, were also similar (data not shown). General screening for behavioral phenotyping was also performed. There was no significant difference between PGAP1+/+ and PGAP1−/− mice with respect to general appearance, posture, gait, spontaneous behaviors, neurological reflexes, eyesight, and olfaction, but PGAP1−/− mice showed growth retardation compared with heterozygous or wild-type mutants, even though weaning and other dietary behavior looked normal (Fig. 4). Retarded growth curves were also observed in PGAP1−/− mutants in another clone (data not shown).

**Male Infertility of PGAP1 Knock-out Mice**—To analyze male fertility, 6 male homozygous mutant mice were mated each with 3 BDF-1 female mice for 8 weeks. Sibling PGAP1+/+ or PGAP1+/− mice showed normal fertility, but none of the 6 PGAP1−/− mice sired a pup despite the presence of vaginal plugs after copulation, clearly indicating male infertility. One male homozygous mutant of another clone underwent the same experiment, and there was only one siring of 4 pups in 2-month mating, although plugs were admitted for each female wild-type mouse mated at least once during the period. Sibling PGAP1+/+ or PGAP1+/− male mouse sired averages of 9.0 or 9.8 pups (total 54 and 49 pups each).

**Knock-out Sperm Cannot Ascend from the Uterus into the Oviduct**—Spermatozoa from PGAP1−/− mice were normal in number and motility (data not shown). Mammalian fertilization consists of multiple successive events, including sperm ascent from the uterus into the oviductal tube, sperm adhesion to the zona-pellucida (ZP), formation of a glycoprotein extracellular matrix surrounding the eggs, and membrane fusion of sperm and eggs. To examine at which step normal fertility is disturbed, firstly, we performed a sperm migration assay. Male mice of each genotype were mated with superovulated female mice for 8 weeks. Sibling PGAP1+/+ or PGAP1+/− male mice sired averages of 9.0 or 9.8 pups (total 54 and 49 pups each). Male mice of each genotype were mated with superovulated female wild-type mice. About 2 h after copulation, oviducts were excised with the connective part of the uterus, fixed, and stained to examine the presence of sperm in the oviductal tubes. Sperm from wild-type or heterozygous mutant mice were able to ascend into the oviduct, but those of homozygous mutant mice could not migrate into the oviductal tube (Fig. 5).

**PGAP1 Knock-out Sperm Scarcely Bind to the ZP**—To examine the ability of mutant sperm to bind to the ZP, we incubated sperm of wild-type or homozygous mutants with eggs in buffer and counted the number of sperm attached to the ZP. Homozygous mutant sperm showed poor binding to the ZP compared with wild-type sperm (WT:KO = 11.9:2.9 sperm per egg, t < 0.0001, unpaired Student’s t test) (Fig. 6). The fertilization rate was examined with two pairs of WT and KO mice as...
described under “Experimental Procedures”: the average rate in the knock-out was 2.7% compared with 51.3% for wild-type sperm. Fusion of the sperm and egg was assessed by a zona-free egg sperm penetration assay (21), and both wild-type and homozygous mutant sperm showed normal fusion (data not shown). Poor sperm binding of knock-out mice was repeated in another PGAP1 knock-out line (WT:KO 1.22:0.1 sperm per egg, t < 0.0001). In the second PGAP1 knock-out mouse line, the average fertilization rate was 27.2% for PGAP1−/− and 82.7% for wild type. In the second experiment, we used the mutant line crossed with AKR mice. A higher rate of fertilization in vitro with homozygous mutants may be due to the difference of genetic background or technical effects. These results indicate that PGAP1 knock-out male mice are infertile because of the inability of their sperm to ascend into the oviduct and bind the ZP.

**Increased Expression Levels of GPI-APs in PGAP1−/− Mice**—Next, we examined the expression in sperm of certain proteins that are presumably closely associated with sperm binding to the ZP, and with sperm migration from the uterus into the oviduct. Knock-out of calmegin (19, 31), ADAM1a (fertilin α) (32), ADAM2 (fertilin β) (33), ADAM3 (cyritestin) (34, 35), and testis-specific ACE (tACE) (36, 37) is reported to result in poor binding of sperm to the ZP. The sperm of calmegin knock-out mice are defective in the expression of ADAM2 and ADAM3 (19, 23), and homozygous ADAM1a mutant mice have ADAM3-deficient sperm. Therefore, we examined the expression of ADAM2, ADAM3, and tACE in testis and sperm. There was no significant reduction of ADAM2, ADAM3, and tACE in testis and sperm (Fig. 7A). Izumo is known as a protein that is indispensable for sperm-egg fusion (25), and its expression in knock-out mice was comparable to that in wild-type mice, consistent with the result that sperm-egg fusion was normal with
knock-out sperm. Notably, the amounts of GPI-APs in sperm were affected to various extents, in contrast to that of DAF in testis, which was normal (Fig. 7A). The amounts of both CD52 and DAF were significantly increased in knock-out mice, whereas CD59b and SPAM-1 (PH-20) were reproducibly increased to lesser extents. To confirm the presence of GPI anchor on DAF and CD59b in sperm and testis from PGAP1 knock-out mice, we assessed the hydrophobicity of these proteins using Triton X-114 partitioning (Fig. 7B). Both proteins from PGAP1 knock-out and wild-type mouse testis and sperm were highly efficiently recovered in the detergent phase, indicating that DAF and CD59b were GPI-anchored in PGAP1 knock-out sperm.

DISCUSSION

We found at least three phenotypes in PGAP1 knock-out mice: a developmental defect (otocephaly), growth retardation, and male infertility. Otocephaly or agnathia of mouse was reported to be caused by the defects of several genes like oto (27) and Otx2 (38–42). How these genes affect the craniofacial formation of mice is still unclear, but Wnt proteins are suggested to be affected by Otx2 (43). In the processes of mouse embryonic development, the embryonic axis is first generated in a proximal-distal direction by embryonic day 5.5 (E5.5). The orientation of the axis is then changed to the anterior-posterior direction (44). This axis conversion requires the function of Otx2, a homeobox transcription factor, which is a murine orthologue of the Drosophila orthodenticle (otd) gene (45). Axis conversion failed in Otx2 knock-out embryos, resulting in otocephaly (38, 39). Through examining the expression pattern of various marker genes for anterior visceral endoderm cells like Lefty or Cerl, it was found that a Wnt antagonist, Dickkopf1 (Dkk1), was not expressed in Otx2 knock-out mice (38, 46, 47). Dkk1 inhibits Wnt-induced Frizzled-LRP5/6 complex formation, degrades β-catenin indirectly, and prevents Wnt signaling (48–52). The Wnt family is a well conserved cysteine-rich glycoprotein family among vertebrates, and it participates in various developmental events during embryogenesis (53, 54). Thus, it is hypothesized that the phenotype of the Otx2 mutant is caused by the malfunction of anterior-posterior axis polarization by up-regulation of Wnt/β-catenin signaling due to Dkk1 deficiency. It is known that Wnt/β-catenin signaling is modulated by division abnormally delayed (Dally)-like proteins (Dlp) in Drosophila (55–61). Dally is one of the glycanic members of Drosophila (56, 62, 63). Glypicans are a family of heparan sulfate proteoglycans attached to the cell surface via a GPI anchor.
Two glypican members, Dally and Dlp have been implicated in Wg signaling (55, 62, 64). In the imaginal disc of Drosophila, Dlp cooperates with Notum, an α/β-hydrolase enzyme with homology to plant pectin acetyl esterases, to down-regulate Wg signaling (57, 65). For this down-regulation, Notum is believed to cleave the GPI portion of Dlp (65). If an acylated GPI anchor is resistant to this cleavage, a higher amount of Dlp might enhance Wg signaling. A similar relationship between Dlp and Notum has not been reported in mammals, but Wnt signaling could be controlled by the same mechanism. The participation of GPI-anchor cleavage in Wnt signaling remains to be elucidated.

Once PGAP1 pups have survived the critical period after birth, most of them showed no behavioral abnormality except growth retardation. The body sizes of three genotypes mice at 18.5 days post coitus weren’t significantly different from each other, suggesting that the impaired growth curve of knock-out mice was caused by postnatal reasons. Further analysis is required to identify the underlying mechanism.

Our experiments revealed that PGAP1 knock-out sperm are defective in binding to the zona pellucida. One can argue that the impaired zona binding property may be derived from the impairment of sperm capacitation process done in vitro. However, the zona binding ability of sperm is evident even in the initial stage of incubation in TYH medium or “capacitating medium.” Together with the result that the sperm from PGAP1 null mice underwent normal acrosome reaction estimated from the normal fusing ability, we presume that the decreased zona binding ability is a characteristic of sperm relating to the change of membrane constitution rather than the impaired sperm capacitation process.

There were higher amounts of several GPI-APs on sperm of PGAP1 knock-out mice as detected by Western blotting. We compared CD48 and DAF expression on the surfaces of mononuclear cells of peripheral blood between wild-type mice and PGAP1 knock-out mice, as well as PGAP1-deficient Chinese hamster ovary cells, but no significant difference was observed (data not shown) (10). Thus, those higher expression levels seem unique to sperm. Many GPI-APs, including CD52, DAF, and CD59, are secreted from the male genital tracts and transferred to the surface of sperm, probably in the form of prostasomes (66–68), and GPI-APs like CD52 and P34H were reported to be transferred from epididymis epithelial cells to the sperm surface in the form of epididymosomes (69). The increased levels of GPI-APs, especially CD52 and DAF, on PGAP1 knock-out sperm are therefore likely due to efficient transfer. A three-“footed” anchor structure of GPI-APs in PGAP1 knock-out mice may have a higher tendency to be transferred to the sperm membrane, or be resistant to lipase (including GPI-cleaving enzyme), resulting in the higher amount of expression detected by Western blotting. Does this cause the infertility? We hypothesize that some GPI-APs should be decreased in the amount for efficient fertilization. Sperm need to undergo several phases of maturation, including capacitation (70, 71). Before capacitation, sperm maturation inhibitory components called decapacitation factors should be removed from the surface of sperm (72, 73). One decapitation factor has been studied by Fraser et al. (72, 74), and its receptor on the sperm surface was reported to be a GPI-AP (75). Such three-footed inhibitory factor receptors might be transferred more in PGAP1-deficient mice than in wild-type mice, hindering sperm-egg interactions. Another possibility is that those GPI-anchored inhibitory components might not be properly removed in the process of sperm maturation. It was reported that ACE is indispensable for fertilization (36, 76) and that the cleavage of GPI-APs by ACE is important for male fertility (77). Three-footed GPI anchors might be resistant to this cleavage. It is also possible that signal transduction through membrane rafts is disturbed. A lack of decacylation of the GPI anchor may abolish the following fatty acid remodeling in the Golgi, which is critical for raft association of GPI-APs (78). This alters the localization of the GPI anchor on the membrane and might change GPI anchor-mediated signal transduction. As a general issue, the resistance to GPI-cleaving enzyme and altered localization and signaling of GPI caused by lack of decacylation could result in phenotypes, including otocephaly.

We found three phenotypes of PGAP1 knock-out mice, but their molecular mechanisms are still under investigation. Further analysis will reveal molecules involved in those phenotypes and the physiological importance of decacylation of GPI.

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