The function of glycosylphosphatidylinositol-anchored sperm hyaluronidase PH-20 in fertilization has long been believed to enable acrosome-intact sperm to pass through the layer of cumulus cells and reach the egg zona pellucida. In this study, we have produced mice carrying a null mutation in the PH-20 gene using homologous recombination. Despite the absence of sperm PH-20, the mutant male mice were still fertile. In vitro fertilization assays showed that mouse sperm lacking PH-20 possess a reduced ability to disperse cumulus cells from the cumulus mass, resulting in delayed fertilization solely at the early stages after insemination. Moreover, SDS-PAGE of sperm extracts and subsequent Western blot analysis revealed the presence of other hyaluronidase(s), except PH-20, presumably within the acrosome of mouse sperm. These data provide evidence that PH-20 is not essential for fertilization, at least in the mouse, suggesting that the other hyaluronidase(s) may play an important role in sperm penetration through the cumulus cell layer and/or the egg zona pellucida, possibly in cooperation with PH-20, although the importance of sperm motility cannot be neglected.

Hyaluronic acid, a polymer consisting of repeating disaccharide units of N-acetyl-d-glucosamine and d-glucuronic acid, is one of the most common glycosaminoglycans present in the extracellular matrix of connective tissues (1–3). Because the structural and functional components in the extracellular matrix maintain the tissue architecture, hyaluronic acid is implicated in many physiological processes, including cell migration, proliferation, and differentiation. Hyaluronidase, which hydrolyzes hyaluronic acid demonstrates the existence of other hyaluronidase(s) except PH-20 in mouse sperm. In particular, a hyaluronic acid-hydrolyzing protein with a size of ~55 kDa is abundantly present in soluble proteins released by A23187-induced acrosome reaction of sperm, including the acrosomal components. Thus, the process governing sperm penetration of the cumulus cell layer needs to be reassessed at least in the mouse model.

**EXPERIMENTAL PROCEDURES**

*Generation of Mutant Mice Lacking PH-20—*Genomic clones encoding the PH-20 gene were isolated from a mouse 129SvJ genomic DNA library in AFXII (Stratagene) using a polymerase chain reaction (PCR)-amplified cDNA fragment as a probe. Of 10 clones isolated, two clones, termed mPHG3 and mPHG6, were used for construction of a targeting vector including a neomycin-resistance (neo) expression cassette flanked by a 1.5-kb genomic region of the PH-20 gene and an HSV-TK cassette (Fig. 1A). The construct was designed to replace a part of exon 2 with the neo expression cassette. Following electroporation of the targeting construct, which had been linearized by digestion with NotI, into mouse D3 embryonic stem (ES) cells, homologous recombinants were selected using G418 and gancyclovir as described previously (16). Five ES cell clones containing the targeted mutation were selected from 504 clones resistant to G418 and gancyclovir and injected into C57BL/6 mouse blastocysts. Chimeric male mice were crossed to C57BL/6 or ICR females (Japan SLC Inc., Shizuoka, Japan) to establish heterozygous mutant lines. Homozygous null mice were obtained by heterozygous mating. Hydrolase activity (9–13). When the enzyme activity of PH-20 is blocked, sperm are incapable of entering into the cumulus cell layer (14). The binding of acrosome-reacted sperm to ZP, known as “secondary sperm-ZP binding,” is also inhibited by antibody against PH-20 (15). Thus, these results imply that PH-20 is bifunctional. The N- and C-terminal domains of PH-20 have been reported to be responsible for hyaluronidase and ZP binding activities, respectively (15).

To explore the role of PH-20 in *in vivo*, using homologous recombination we have produced male mice carrying a disruptive mutation in the PH-20 gene. The mice lacking PH-20 are still fertile, providing evidence that PH-20 is not essential for the penetration of sperm through the cumulus cell layer. Moreover, SDS-polyacrylamide gel electrophoresis (PAGE) in the presence of hyaluronic acid demonstrates the existence of other hyaluronidase(s) except PH-20 in mouse sperm. In particular, a hyaluronic acid-hydrolyzing protein with a size of ~55 kDa is abundantly present in soluble proteins released by A23187-induced acrosome reaction of sperm, including the acrosomal components. Thus, the process governing sperm penetration of the cumulus cell layer needs to be reassessed at least in the mouse model.

**Biot Hybridization—**Genomic DNA was prepared from mouse tail, digested by *Bgl* II, separated by agarose gel electrophoresis, and transferred onto Hybond-N*⁺* nylon membranes (Amersham Biosciences). Total cellular RNA was also separated by agarose gel electrophoresis and transferred onto the nylon membranes. The blots were probed with 32P-labeled DNA fragments and analyzed by a BAS-1800II Bio-Image analyzer (Fuji Photo Film, Tokyo) as described previously (17).

**Preparation of Antibody—**Recombinant mouse PH-20 produced in
fractionation of sperm proteolytic activity was measured by the colorimetric method using Alcian Blue 8 GX (Sigma), as described previously (22). Following electrophoresis, gels were washed with 50 mM sodium acetate buffer, pH 6.0, containing 150 mM NaCl and 3% sodium dodecyl sulfate (SDS)–PAGE under nonreducing conditions as described previously (22). Following electrophoresis, gels were visualized as transparencies against a blue background by staining the gels with 0.5% Alcian Blue 8 GX and Coomassie Brilliant Blue R250 (Sigma).

**Preparation of Protein Extracts**—Fresh cauda epididymal sperm were collected in a modified Krebs-Ringer bicarbonate solution (TYH medium) (19) containing 50 mM Hepes, washed with phosphate-buffered saline (PBS) by centrifugation at 800 × g for 10 min, and extracted in PBS containing 30 mM n-octyl-β-D-glucopyranoside (Dojindo, Kumamoto, Japan), 1 mM EDTA, 1 mM benzamidine/HCl, 1 mM phenylmethanesulfonyl fluoride, leupeptin (1 μg/ml), and pepstatin A (1 μg/ml), as described above. After centrifugation at 13,000 × g for 10 min, the supernatant was used as a source of membranous proteins on the plasma and outer acrosomal membranes fused during the acrosome reaction (MFA fraction). Proteins were also extracted from acrosome-intact and acrosome-reacted sperm with the same PBS containing n-octyl-β-D-glucopyranoside and protease inhibitors (AI and AR fractions, respectively). The concentrations of proteins in the four fractions were determined using a Coomassie protein assay reagent kit (Pierce).

**SDS-PAGE and Western Blot Analysis**—Proteins were separated by SDS-PAGE under nonreducing conditions and transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore). After blocking with 2% skim milk, the blots were incubated with affinity-purified anti-mouse PH-20 antibody at room temperature for 2 h and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) for 1 h. The immunoreactive proteins were detected by an ECL Western blotting detection kit (Amersham Biosciences). To detect proteins exhibiting hyaluronidase activity, SDS-PAGE in the presence of 0.1% human umbilical cord hyaluronidase was carried out under nonreducing conditions as described previously (22). Following electrophoresis, gels were washed with 50 mM sodium acetate buffer, pH 6.0, containing 150 mM NaCl and 3% Triton X-100 at room temperature for 2 h to remove SDS and then incubated in the same buffer without Triton X-100 at 37°C overnight. The hyaluronic acid–hydrolizing proteins were visualized as transparencies against a blue background by staining the gels with 0.5% Alcian Blue 8 GX and Coomassie Brilliant Blue R250 (Sigma).

**Fertilization Assays in Vitro**—Female ICR mice (8 weeks old) were superovulated by intraperitoneal injection of pregnant mare’s serum gonadotropin (5 units) followed by human chorionic gonadotropin (5 units) 48 h later. Eggs were collected from the oviductal ampulla of the superovulated mice at 15–16 h after injection of human chorionic gonadotropin and placed into an 0.2-ml drop of TYH medium (19) covered with mineral oil. Fresh cauda epididymal sperm from 3-month-old mice were capacitated by incubation for 2 h in a 0.2-ml drop of TYH medium at 37°C under 5% CO₂ in air. An aliquot of the capacitated sperm suspension (1.5 × 10⁶ sperm/ml) was mixed with the eggs in TYH medium. The eggs and sperm were incubated for 0.5–6 h at 37°C under 5% CO₂ in air. At the end of incubation, the eggs were briefly treated with bovine testicular hyaluronidase, fixed with glutaraldehyde, and then mounted on slides for whole-mount preparation. The eggs were stained with 0.25% lacmoid for the assessment of in vitro fertilization as described (16, 23).

**Fertilization assay in vitro**—For competitive fertilization assay in vitro, eggs with or without associated cumulus cells were incubated with an equally mixed suspension of wild-type and PH-20-deficient mouse sperm. The final concentrations of sperm were 1.5 × 10⁷ and 5.0 × 10⁷ sperm/ml for the cumulus-intact and cumulus-free eggs, respectively. Following incubation at 37°C for 6 h, the fertilized eggs were washed twice with a modified simplex-optimized medium, kSOM/AA (24), and incubated at 37°C for 96 h under 5% CO₂ in air. Each of the developing embryos was lysed in a 0.04% SDS solution (2 μl) containing 200 mM NaOH by boiling for 15 min, and was then used as a template for PCR amplification. The PCR reaction was carried out in a 40-μl mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, dATP, dGTP, dCTP, and dTTP at each 0.2 mM, the oligonucleotide primers each at 1 μM, template DNA, and 1 unit of Gene Taq DNA polymerase (Nippon Gene, Toyama, Japan), using MHA3 (5'-TTGAAAGTCTCAATC GACAAAGC-3') and MHA16 (5'-GGATTCTCAGGATGACGACGAC-3'), respectively.
were examined for Ph20 (mean 30312 Ph20 a probe (Fig. 1). An immunoreactive 52-kDa protein corresponding to PH-20 was demonstrated no significant difference of the shapes, numbers, and sizes of testicular germ cells and epididymal sperm among Ph20+/−, Ph20+/+, and Ph20−/− mice (data not shown). Both the formation of copulation plugs in mated female mice and the motility of cauda epididymal sperm were also normal in the heterozygotes and homozygotes. Moreover, male Ph20+/− mice showed normal fertility and produced an average litter size (means ± S.E. = 13.8 ± 0.4, 13.6 ± 1.4, and 12.2 ± 0.8 for 5, 5, and 21 litters in Ph20+/+, Ph20+/−, and Ph20−/− mice, respectively). Essentially similar results were obtained in the mouse lines derived from two independent ES clones. Therefore, these results provide evidence that PH-20 is not essential for fertilization at least in the mouse. Female Ph20−/− mice also exhibited normal fertility.

To examine whether the absence of PH-20 affects the process of sperm penetration through the layer of cumulus cells, in vitro fertilization analysis was carried out using capacitated cauda epididymal sperm. We have categorized the status of the mass of cumulus cells surrounding an egg into four patterns (patterns a, b, c, and d) to monitor successive dispersal of cumulus cells from the mass (Fig. 2A). When the cumulus cell mass was incubated in the absence of sperm, cumulus cells were spontaneously broken away from the mass. However, the eggs were still associated with many cumulus cells (patterns a, b, and c) in the masses even after the incubation for 6 h (Fig. 2B). Ph20−/+ mouse sperm readily dispersed cumulus cells from the mass, and ~70% of the eggs completely lost cumulus cells (pattern d) 3 h after insemination. As compared with the wild-type mouse, the Ph20−/− mouse showed a remarkable delay in the dispersal of cumulus cells from the mass. The ratio of the pattern d cumulus cell mass was less than 20% in the Ph20−/− mouse at 3 h after insemination. Thus, the delayed dispersal of cumulus cells may reflect the reduced ability of Ph20−/− mouse sperm to hydrolize hyaluronic acid in the extracellular matrix of cumulus cells.

As shown in Fig. 3A, an in vitro fertilization assay confirmed the fertility of PH20−/− mouse sperm. Although Ph20+/+ and Ph20−/− mouse sperm equally fertilized the eggs 3 h after insemination, the rate of fertilization in Ph20−/− mouse sperm was significantly lower than that in Ph20+/+ mouse sperm solely at the early stages (1 and 2 h) after insemination. To verify the delayed fertilization of Ph20−/− mouse sperm, eggs with or without associated cumulus cells were inseminated with an equally mixed suspension of Ph20+/+ and Ph20−/− mouse sperm. The fertilized eggs with male and female pronu-
In vitro fertilization embryos developed from fertilized eggs. Cauda epididymal sperm of the wild-type (Ph20+/+), open column) and PH-20-deficient (Ph20−/−, shaded column) mice were capacitated and incubated with metaphase II-arrested, cumulus-intact eggs for 1, 2, or 3 h. After the incubation, the eggs were washed with TYH medium (see Ref. 19), treated briefly with bovine testicular hyaluronidase, washed, and then re-incubated for 5, 4, or 3 h (total incubation time = 9 h). The eggs with female and male pronuclei were defined as “fertilized eggs.” The numbers in the columns represent the numbers of eggs examined. B, PCR analysis of genomic DNA of the embryos developed from fertilized eggs in vitro. Metaphase II-arrested eggs with (235 eggs) or without associated cumulus cells (268 eggs) were incubated with an equally mixed suspension of Ph20+/+ and Ph20−/− mouse sperm. After incubation for 6 h, the fertilized eggs were further incubated for 96 h. Genomic DNA was prepared from each of the developing embryos and then used as a template for PCR amplification. Two DNA fragments with the sizes of 254 and 145 bp, originated from the null-mutated (KO) and wild-type (WT) alleles, respectively, were detected by PAGE. The patterns of Ph20+/+ mouse tail (lane T) and eight developing embryos (lanes 1–8) are indicated. C, competitive fertilization assay of cauda epididymal sperm with cumulus-intact and cumulus-free eggs. Metaphase II-arrested eggs with or without associated cumulus cells were incubated with an equally mixed suspension of Ph20+/+ (open column) and Ph20−/− (shaded column) mouse sperm, and the genotype of each of the developing embryos was assessed by PCR analysis as described in B above.

The nuclei were then developed into embryos in vitro, and the genotype of each of the embryos was assessed by PCR. Two DNA fragments with the sizes of 254 and 145 nucleotides, which were PCR-amplified from the null-mutated and wild-type alleles, respectively, were detected (Fig. 3B). When the cumulus-intact eggs were used, Ph20+/+ mouse sperm were approximately three times slower to fertilize the eggs than Ph20−/− mouse sperm (Fig. 3C). In the cumulus-free eggs, the fertilization rate in Ph20+/− mouse sperm was still slow but close to that in Ph20+/+ mouse sperm. These data imply that the reduced fertilization rate in Ph20−/− mouse sperm may be due to the delay of the sperm penetration through the cumulus cell layer.

Despite the time delay, Ph20−/− mouse sperm are indeed capable of penetrating the layer of cumulus cells. This fact raises a possibility that other hyaluronidase(s) besides PH-20 may be present in mouse sperm and may participate in the sperm penetration through the cumulus layer, possibly in cooperation with PH-20. To ascertain this possibility, acrosome-intact sperm were extracted from cumulus-free eggs (Fig. 4A). The activities in the AI, AR, SPA, and MPA fractions from the Ph20+/+ and Ph20−/− mice, separated by SDS-PAGE in the absence or presence (Hyase activity) of hyaluronic acid under nonreducing conditions, and analyzed by Western blotting using affinity-purified anti-mouse PH-20 antibody. As a control, affinity-purified antibody against an acrosomal proacrosin-binding protein, sp32, was also used.

This study demonstrates both a partial contribution of mouse PH-20 toward the sperm penetration through the layer of cumulus cells (Figs. 2 and 3), and the presence of other hyaluronidase(s) besides PH-20 in mouse sperm (Fig. 4). PH-20 has long been thought to be the sole hyaluronidase involved in consistent with the experimental data obtained by Western blot analysis (Fig. 1D).

To examine the location of a 55-kDa protein(s) and PH-20 exhibiting hyaluronidase activity in sperm, four protein fractions (AI, AR, SPA, and MPA) were prepared from cauda epididymal sperm of Ph20+/+ and Ph20−/− mice and analyzed by Western blotting using affinity-purified anti-mouse PH-20 antibody (Fig. 4C). Affinity-purified antibody against an acrosomal proacrosin-binding protein, sp32 (26, 27), was also used as a control. In the Ph20−/− mouse, only the SPA fraction contained no PH-20, whereas the 55-kDa protein was abundantly present in the SPA fraction. In addition, PH-20 was absent in the AI, AR, and MPA fractions from the Ph20−/− mouse. These data clearly demonstrate the presence of other hyaluronidase(s) besides PH-20 presumably within the acrosome of mouse sperm. It should be noted that a very small amount of the hyaluronidase activity resulting from the 55-kDa protein(s) is also found in AR fraction. This may be due to the presence of contaminating acrosome-intact sperm in the AR fraction, because only 80–90% of sperm were acrosome-reacted by calcium ionophore A23187 under the conditions employed in the present study. Indeed, acrosomal protein sp32 is slightly but significantly detectable in the AR fraction (Fig. 4C).

**DISCUSSION**

This study demonstrates both a partial contribution of mouse PH-20 toward the sperm penetration through the layer of cumulus cells (Figs. 2 and 3), and the presence of other hyaluronidase(s) besides PH-20 in mouse sperm (Fig. 4). PH-20 has long been thought to be the sole hyaluronidase involved in...
sperm penetration through the cumulus cell layer, because other sperm hyaluronidases have not been characterized well. Our results indicate that a 55-kDa protein exhibiting hyaluronidase activity is abundantly released by calcium ionophore-induced acrosome reaction presumably from the acrosome (Fig. 4). If the acrosome reaction does not occur until acrosome-intact sperm reach the egg ZP, the 55-kDa hyaluronidase may be functional in the sperm/ZP interactions, including secondary sperm-ZP binding. It is also speculated that the motility may be the most important factor in the sperm penetration of the cumulus layer, if hyaluronidases other than PH-20 are absent on the sperm membrane. Thus, the other sperm hyaluronidases remain to be characterized.

Mice carrying either of two Robertsonian translocations on chromosome 6, Rb(6.16) and Rb(6.15), have been reported as showing a significant transmission ratio distortion in the progeny (transmission ratios of 3:6:1 and 2:4:1, respectively) (28–30), as found in the mice carrying different t alleles (31). The impaired fertility of Rb-bearing sperm seems to be the result of a decreased amount of hyaluronidase activity on the sperm membrane (32). The gene encoding sperm adhesion molecule 1, Spam1, identical to PH-20, is a candidate gene involved in the sperm dysfunction leading to transmission ratio distortion in the Rb(6.16) and Rb(6.15) mice (33). In the present study, the impaired ability of PH20−/− mouse sperm to fertilize cumulus-intact eggs in vitro (Fig. 3) is apparently consistent with the dysfunction of Rb-bearing mouse sperm. However, PH20−/− male mice produce a normal average litter size, in contrast with the Rb homozygous mice. These data imply that the dysfunction of Rb-bearing mouse sperm may not be ascribed solely to the reduced amount of sperm Spam1/PH-20.

The human and mouse genomes have been reported to possess six hyaluronidase-like genes, each three genes of which form a cluster on the chromosomes (34). In the mouse, three genes corresponding to the human HYALP1, HYAL4, and PH-20/SPAM1 genes are clustered within the region of ~65 kbp on mouse chromosome 6 A2 (34). As far as we have examined, the mouse HYALP1 and PH-20/SPAM1 genes are both expressed exclusively in testicular tissues, whereas expression of the mouse HYAL4 gene in the testis is barely detectable. We have also found the presence of an additional hyaluronidase-like gene (tentatively termed HYAL5) that is localized almost 100 kbp away from the PH-20/SPAM1 gene on the mouse chromosome 6. It has been demonstrated that multiple mutations occur in the PH-20/SPAM1 gene of Rb(6.16) and Rb(6.15) mice because of recombination suppression near the Rb junctions (35), although no direct evidence has been provided that these mutations are responsible for the reduction in the hyaluronidase activity and gene expression. Even so, other point mutation(s) that affect the enzyme activity may occur in the HYALP1 and HYAL5 genes because of the localization adjacent to the PH-20/SPAM1 gene on chromosome 6. Thus, additional experiments concerning possible mutations in the HYALP1 and HYAL5 genes would seem to be required. Moreover, it is important to ascertain whether these gene products are indeed present in mouse sperm.

The reduced rate of PH20−/− mouse sperm in vitro fertilization with cumulus-intact eggs (Fig. 3) appears to be ascribable solely to the delayed penetration through the layer of cumulus cells. However, there is a possibility that an incomplete interaction between PH20−/− sperm and egg ZP, because of the absence of PH-20, may result in the reduced fertilization rate. Our preliminary experiments indicate that no significant difference of ability to bind cumulus-free, ZP-intact eggs 30 min after insemination was observed among PH20−/−, PH20−/−, and PH20−/− mouse sperm (numbers of sperm bound to ZP/egg = 8.8 ± 1.6, 11.3 ± 1.8, and 11.5 ± 2.2, respectively). These data appear to weaken the possible importance of PH-20 in the sperm/ZP interactions, although we have not yet examined the effects of mouse sperm lacking PH-20 on the secondary sperm-ZP binding using “live” acrosome-reacted sperm.

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