Identification of a hyaluronidase, Hyal5, involved in penetration of mouse sperm through cumulus mass

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A glycosylphosphatidylinositol (GPI)-anchored hyaluronidase, PH-20, on the sperm surface has long been believed to assist sperm penetration through the cumulus mass surrounding the eggs. However, mouse sperm lacking PH-20 were still capable of penetrating the cumulus mass despite a delayed dispersal of cumulus cells. Intriguingly, a 55-kDa hyaluronan-hydrolyzing protein was abundantly present in wild-type and PH-20-deficient mouse sperm. In this study, we purified the 55-kDa mouse protein from soluble protein extracts released from epididymal sperm by acrosome reaction and identified as a hyaluronidase, Hyal5. *Hyal5* was exclusively expressed in the testis and formed a 160-kbp gene cluster together with *Hyalp1*, *Hyal4*, and *Ph-20* on mouse chromosome 6. Hyal5 was a single-chain hyaluronidase present on the plasma and acrosomal membranes of sperm presumably as a GPI-anchored protein. Moreover, hyaluronan zymography revealed that Hyal5 is enzymatically active in the pH range 5–7 and inactive at pH 3 and 4. Both Hyal5-enriched PH-20-free soluble protein extracts and PH-20-deficient mouse sperm were capable of dispersing cumulus cells from the cumulus mass. Cumulus cell dispersal was strongly inhibited by the presence of a hyaluronidase inhibitor, apigenin. These results suggest that in the mouse, Hyal5 may function principally as a ''cumulus matrix depolymerase'' in the sperm penetration through the cumulus mass and in the local hyaluronan hydrolysis near or on the surface of the egg zona pellucida to enable the proximal region of sperm tail to move freely. PH-20 may compensate in part for the functional roles of Hyal5.

cumulus cells | fertilization | PH-20

Hyaluronan (hyaluronic acid or hyaluronate), a polysaccharide consisting of a repeating disaccharide unit of *N*-acetyl-D-glucosamine and D-glucuronic acid, is a component of the extracellular matrix of vertebrate tissues, particularly of soft connective tissues (1–3). This glycosaminoglycan is thought to participate in many biological processes, because the hyaluronan level is markedly elevated during embryogenesis, cell migration, wound healing, malignant transformation, and tissue turnover. Hyaluronidase responsible for degradation of hyaluronan is also distributed widely in mammals, insects, leeches, and bacteria (2–4). In the human and mouse, at least six hyaluronidase-like genes are clustered as two tightly linked triplets on two chromosomes (5, 6): *HYAL1*, *HYAL2*, and *HYAL3* on human chromosome 3p21 (*Hyal1*, *Hyal2*, and *Hyal3* on mouse chromosome 9F1-F2) and *HYAL4*, *PH-20/SPAM1*, and *HYALP1* on human chromosome 7q31 (*Hyal4*, *Ph-20*, and *Hyalp1* on mouse chromosome 6A2).

Mammalian fertilization requires sperm to penetrate the cumulus mass surrounding the eggs to reach the zona pellucida (ZP) (7–10). Because cumulus cells are embedded in the extracellular matrix abundant in hyaluronan (11), a glycosylphosphatidylinositol (GPI)-anchored sperm hyaluronidase, PH-20, has long been believed to catalyze the hyaluronan degradation, thus enabling acrosome-intact (AI) sperm to penetrate the cumulus mass (9, 12–17). A possible involvement of PH-20 in the binding of acrosome-reacted (AR) sperm to ZP, known as secondary sperm/ZP binding, has been also suggested (18). However, male mice lacking PH-20 ($Ph20^{-/-}$) are still fertile, although epididymal sperm of $Ph20^{-/-}$ mice show a delayed dispersal of cumulus cells from the cumulus mass *in vitro* (19). In addition, no significant difference in sperm binding to the ZP is found between wild-type and $Ph20^{-/-}$ mice. Noteworthy is that a 55-kDa protein exhibiting hyaluronan-hydrolyzing activity, besides 52-kDa PH-20, is abundantly present in epididymal sperm of mice (19). Thus, the 55-kDa hyaluronidase may play a crucial role in the sperm penetration through the cumulus mass, possibly in cooperation with PH-20.

In this study, we purified the 55-kDa hyaluronan-hydrolyzing protein from mouse epididymal sperm. Peptide MS analysis reveals that the purified protein is identical to a hyaluronidase, termed Hyal5, specifically expressed in the testis. Hyal5 is likely to be a GPI-anchored single-chain polypeptide localized on both plasma and acrosomal membranes of sperm. A Hyal5-enriched protein fraction prepared from $Ph20^{-/-}$ mouse sperm is capable of dispersing cumulus cells from the cumulus mass. Moreover, a hyaluronidase inhibitor, apigenin, effectively prevents $Ph20^{-/-}$ mouse sperm from dispersing cumulus cells. These results imply the principal roles of Hyal5 in sperm penetration through the cumulus mass and probably in local hyaluronan hydrolysis near or on the ZP surface.

Materials and Methods

Preparation of Sperm Protein Extracts. Fresh cauda epididymal sperm were collected in a modified Krebs-Ringer bicarbonate solution (TYH medium) (20), washed with PBS by centrifugation at 800 × *g* for 10 min, and extracted in 20 mM Tris·HCl, pH 7.4, containing 1% Triton X-100, 0.15 M NaCl, and 1% protease inhibitor mixture (Sigma–Aldrich) on ice for 6 h. The sperm suspension was centrifuged at 13,000 × *g* for 10 min, and the supernatant solution was used as proteins from AI sperm (AI fraction). Protein extracts were also prepared from AR sperm, as described (19, 21). Briefly, epididymal sperm (5×10^7 cells/ml) in TYH medium were induced to undergo acrosome reaction by addition of calcium ionophore A23187 (Sigma–Aldrich) at a final concentration of 5 μg/ml followed by incubation at 37°C for 1 h under 5% CO_2 in air. After centrifugation, the supernatant was recentrifuged in a Beckman Optima MAX-E ultracentrifuge using a TLA-100.3 rotor at 100,000 × *g* for 90 min. The resulting supernatant was used as soluble proteins released by the A23187-induced acrosome reaction, including acrosomal components (SPA fraction). The precipitate obtained by ultracentrifugation was washed with PBS, extracted at 4°C for 6 h in PBS containing 1% Triton X-100, 1 mM EDTA, and 1% protease inhibitor mixture, and

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Abbreviations: ZP, zona pellucida; GPI, glycosylphosphatidylinositol; AR, acrosome reacted; AI, acrosome intact; PI-PLC, phosphatidylinositol phospholipase C.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AB085680).

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then centrifugation at 13,000 × *g* for 10 min. The supernatant was used as membranous proteins on the plasma and outer acrosomal membranes fused during the acrosome reaction (MPA fraction). AR sperm were extracted in the above Tris·HCl buffer and used as proteins from AR sperm (AR fraction). The protein concentration was determined by using a Coomassie protein assay reagent kit (Pierce).

Purification of Hyaluronan-Hydrolyzing Protein. The SPA fraction was prepared from AR sperm (1×10^9 cells), as described above, and applied onto a HiTrap heparin HP column (Amersham Pharmacia Biosciences) that had been equilibrated with 20 mM Tris·HCl (pH 7.5). The column was washed with the same buffer (10 ml), and proteins were eluted from the column with a linear gradient of 0–0.5 M NaCl in the same buffer (15 ml) at a flow rate of 0.5 ml/min. Aliquots of each fraction (0.5 ml) were analyzed by SDS/PAGE in the presence of hyaluronan, as described below. Fractions containing the hyaluronan-hydrolyzing protein were combined, concentrated by using a Vivaspin 500 (Vivascience, Hannover, Germany), and subjected to 2D PAGE (22).

Peptide MS Analysis. After 2D PAGE, protein spots on the gels were cut, digested with trypsin (Promega, V5111), and analyzed by an AXIMA MALDI-TOF MS (Shimadzu, Kyoto) according to the manufacturer's protocol. Proteins were identified by the "mass fingerprinting" method using a MASCOT software (Infocom, Tokyo).

Zymography. Proteins exhibiting hyaluronidase activity were visualized by SDS/PAGE in the presence of 0.01% human umbilical cord hyaluronan (Sigma–Aldrich) under nonreducing conditions, as described (19, 23). After electrophoresis, gels were washed with 50 mM sodium acetate buffer, pH 6.0, containing 0.15 M NaCl and 3% Triton X-100 at room temperature for 2 h to remove SDS and then incubated in the same buffer free of Triton X-100 at 37°C overnight. The hyaluronan-hydrolyzing proteins were detected as transparent bands against a blue background by staining the gels with 0.5% Alcian Blue 8 GX and Coomassie brilliant blue R250 (Sigma–Aldrich).

Immunoblot Analysis. Proteins were separated by SDS/PAGE and transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore). After blocking with 2% skim milk in 50 mM Tris·HCl, pH 7.5, containing 0.15 M NaCl and 0.1% Tween-20, the blots were probed by primary antibodies and then incubated with secondary antibodies conjugated with horseradish peroxidase. The immunoreactive proteins were visualized by an enhanced chemiluminescence Western blotting detection kit (Amersham Pharmacia Biosciences), as described (24). The intensities of the immunoreactive protein bands were quantified by using Fuji Film SCIENCE LAB IMAGE GAUGE software (Version 3.4).

Biotinylation of Sperm Surface Proteins. Cauda epididymal sperm (2.5×10^7 cells/ml) were washed twice with PBS by centrifugation at 1,000 × *g* for 5 min, incubated at room temperature for 30 min in PBS containing 1 mM sulfo-NHS-LC-biotin (Pierce), and washed twice with PBS (25, 26). The biotinylated proteins were extracted in 20 mM Tris·HCl, pH 7.4, containing 1% Triton X-100, 0.15 M NaCl, and 1% protease inhibitor mixture on ice for 2 h. After centrifugation at 13,000 × *g* for 10 min, proteins in the supernatant were analyzed by immunoblotting, as described above.

Phosphatidylinositol Phospholipase C (PI-PLC) Treatment. Epididymal sperm (5×10^6 cells) were dispersed in TYH medium for 10 min, washed three times with cold PBS, and suspended in PBS (0.1 ml) containing 1 unit of *Bacillus cereus* PI-PLC (Biomol, Plymouth, PA). The mixture was incubated at 37°C for 1 h and centrifuged. Proteins in the supernatant were subjected to immunoblot analysis.

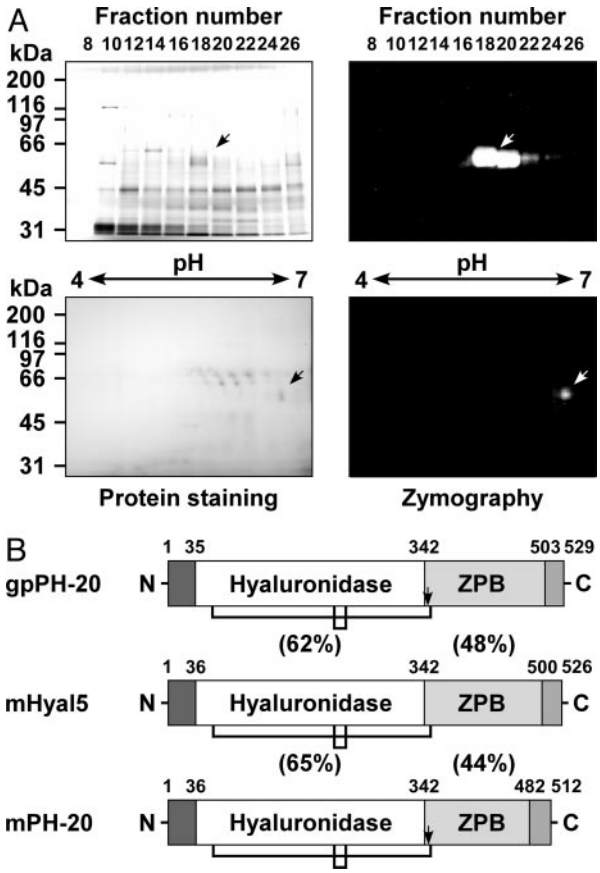

Fig. 1. A 55-kDa hyaluronan-hydrolyzing protein from mouse epididymal sperm is identical to Hyal5. (*A*) Purification of a 55-kDa sperm protein. Soluble sperm proteins (SPA fraction) released by calcium ionophore-induced acrosome reaction were applied onto a HiTrap heparin HP column (*Upper*). Proteins were eluted with a linear gradient of 0–0.5 M NaCl and analyzed by SDS/PAGE in the presence of hyaluronan. Fractions containing the 55-kDa hyaluronan-hydrolyzing protein (arrows) were combined and subjected to 2D PAGE (*Lower*). The 55-kDa protein was identified as a hyaluronidase-like protein, Hyal5, by peptide MS analysis. (*B*) Schematic representation of guinea pig PH-20 (gpPH-20), mouse PH-20 (mPH-20), and mouse Hyal5 (mHyal5). These three proteins contain four putative domains: the N-terminal signal peptide domain (darkly shaded boxes), the catalytic domain as hyaluronidase, the ZP-binding domain (ZPB), and the C-terminal recognition domain for attachment to GPI (lightly shaded boxes). Two disulfide-bond arrangements are assigned by the sequence similarities of mPH-20 and mHyal5 with gpPH-20. The sequence identities of the catalytic (hyaluronidase) and ZP-binding domains between mHyal5 and gpPH-20 or mPH-20 are also indicated (percentages in parentheses). Arrows represent the position of the endoproteolytic cleavage site determined in gpPH-20 and mPH-20.

Identification of ZP-Binding Proteins. Female ICR mice (8 weeks old, SLC Japan, Shizuoka, Japan) were superovulated by i.p. injection of pregnant mare's serum gonadotropin (5 units, Teikokuzoki, Tokyo) followed by human chorionic gonadotropin (5 units, Teikokuzoki) 48 h later. Cumulus mass containing eggs was collected from the oviductal ampulla of the superovulated mice 14 h after injection of human chorionic gonadotropin, placed in a 50-μl drop of TYH medium covered with mineral oil, treated with bovine hyaluronidase (3 units/ml, Sigma–Aldrich), and washed with TYH medium. The cumulus-free eggs (120 cells) were biotinylated in PBS containing 1 mM sulfo-NHS-LC-biotin at room temperature for 30 min. The biotinylated ZP was solubilized in acid Tyrode's solution (Sigma–Aldrich) by pipetting, incubated with 0.2 ml of ImmunoPure immobilized streptavidin beads (50% slurry, Pierce) in 20 mM Tris·HCl, pH 7.4, containing 1% Triton X-100 and 150

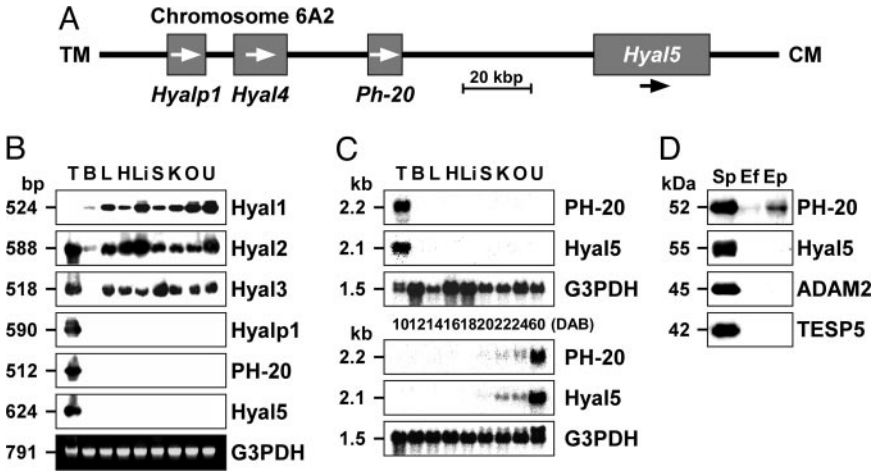

Fig. 2. Organization and expression of mouse *Hyal5*. (*A*) Chromosomal orientation of hyaluronidase genes. Four mouse genes, *Hyalp1*, *Hyal4*, *Ph-20*, and *Hyal5*, are located on chromosome 6A2. Arrows indicate the protein-coding directions of these four genes. TM, telomere; CM, centromere. (*B*) RT-PCR analysis. A portion of first-strand cDNAs synthesized from total cellular RNAs of mouse testis (T), brain (B), lung (L), heart (H), liver (Li), spleen (S), kidney (K), ovary (O), and uterus (U) was subjected to PCR followed by Southern blot analysis using specific internal ^{32}P-labeled DNAs as probes. In glyceraldehyde-3-phosphate dehydrogenase (G3PDH), the PCR-amplified DNA fragments were stained with ethidium bromide. Note that no DNA band was detectable in the nine tissues when PCR was carried out by using a set of oligonucleotide primers specific for Hyal4 (data not shown). (*C*) Northern blot analysis. Total RNAs (10 μg each) of various mouse tissues and the testes of mice at 10–60 days after birth (DAB) were subjected to Northern blot analysis, using ^{32}P-labeled DNA fragments of PH-20, Hyal5, and G3PDH as probes. (*D*) Immunoblot analysis. Protein extracts (3 μg each) of mouse epididymal sperm (Sp), luminal fluids (Ef), and tissues (Ep) were analyzed by immunoblotting, using antibodies against PH-20, Hyal5, ADAM2, and TESP5. The epididymal fluids and tissues contain only PH-20.

mM NaCl at 4°C for 2 h, and washed three times with the same buffer. Protein extracts (0.5 mg/ml) of cauda epididymal sperm in the same buffer were mixed with the streptavidin beads, and the mixture was rotated at 4°C for 2 h. After washing thoroughly with PBS by centrifugation, the precipitate was treated with an SDS sample buffer and subjected to immunoblot analysis. All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals in University of Tsukuba.

Dispersal of Cumulus Cells *in Vitro*. Cumulus mass containing eggs was prepared as described above. 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% CO_2 in air. An aliquot of the capacitated sperm suspension (1.5×10^5 cells/ml) or SPA fraction of sperm extracts (3 mg of protein/ml) was mixed with the cumulus mass in TYH medium (50 μl) containing apigenin. The mixture was incubated at 37°C for 30 or 180 min under 5% CO_2 in air and then observed under an Olympus (Tokyo) IX71 microscope equipped with a DP-12 camera, as described (27).

Antibodies, Blot Hybridization, and RT-PCR. Details are available in *Supporting Text*, which is published as supporting information on the PNAS web site.

Results

We have previously demonstrated that a 55-kDa hyaluronan-hydrolyzing protein is abundantly present in soluble proteins (SPA fraction) released by A23187-induced acrosome reaction of epididymal sperm (19). Thus, the SPA fraction of wild-type mouse sperm was used as a purification source of the 55-kDa hyaluronan-hydrolyzing protein. The 55-kDa protein was eluted from a HiTrap heparin HP column with ≈0.3 M NaCl and was further separated from other proteins by 2D PAGE (Fig. 1*A*). Peptide MS analysis revealed that the 55-kDa hyaluronan-hydrolyzing protein is identical to a hyaluronidase-like protein, Hyal5 (GenBank/European Bioinformatics Institute accession nos. AK017112 and AB085680).

Guinea pig PH-20 is known to be a GPI-anchored protein containing four domains (8, 17, 18): the signal peptide domain at the N terminus, the catalytic domain as hyaluronidase, the ZP-binding domain, and the recognition domain for attachment to GPI at the C terminus (Fig. 1*B*). The DNA-derived amino acid sequence indicated that mouse Hyal5 is initially synthesized as a single-chain polypeptide of 526 amino acids with a calculated molecular mass of 60,807 Da (Fig. 1*B*; see also Fig. 6, which is published as supporting information on the PNAS web site). On the basis of the sequence similarity to guinea pig and mouse PH-20, the 36- and 26-residue sequences of mouse Hyal5 at the N and C termini were predicted to function as the signal sequences for a nascent protein destined for initial transfer to the endoplasmic reticulum and for the GPI attachment, respectively. The catalytic (hyaluronidase) and ZP-binding domains of Hyal5, which were assigned to the 306- and 158-residue sequences at positions 37–342 and 343–500 (Fig. 1*B*), shared noticeable degrees of sequence identities (62% and 48%) with the corresponding domains of guinea pig PH-20 (65% and 44% for mouse PH-20, respectively). The locations of 12 Cys residues in Hyal5 were completely conserved with those in guinea pig and mouse PH-20. In addition, the entire sequence of Hyal5 is relatively divergent to those of mouse Hyal1, Hyal2, Hyal4, and Hyalp1 (32–36% identities).

When we previously screened a mouse 129/SvJ genomic DNA library by using a DNA fragment coding for PH-20 as a probe, genomic clones encoding Hyal5 were identified together with those encoding PH-20 (19). However, the details of mouse Hyal5 have been largely unknown at the molecular and functional levels (19, 28). Sequencing analysis of these three clones indicated that *Hyal5* is ≈34 kbp in length and contains four exons interrupted by three introns (Fig. 7, which is published as supporting information on the PNAS web site). The translation initiator codon, ATG, was encoded within the second exon of *Hyal5*. A computer-aided search on the National Center for Biotechnology Information Genomic Biology database (www.ncbi.nlm.nih.gov/genomes) revealed that *Hyal5* is localized 57 kbp away from *Ph-20* on mouse chromosome 6A2 (Fig. 2*A*). In addition, Southern blot analysis verified the presence of *Hyal5* on the mouse genome as a single-copy gene (Fig. 7). To examine expression of *Hyal5* in nine mouse tissues, RT-PCR analysis was carried out by using total cellular RNAs as templates (Fig. 2*B*). *Hyal1*, *Hyal2*, and *Hyal3* were expressed in all tissues

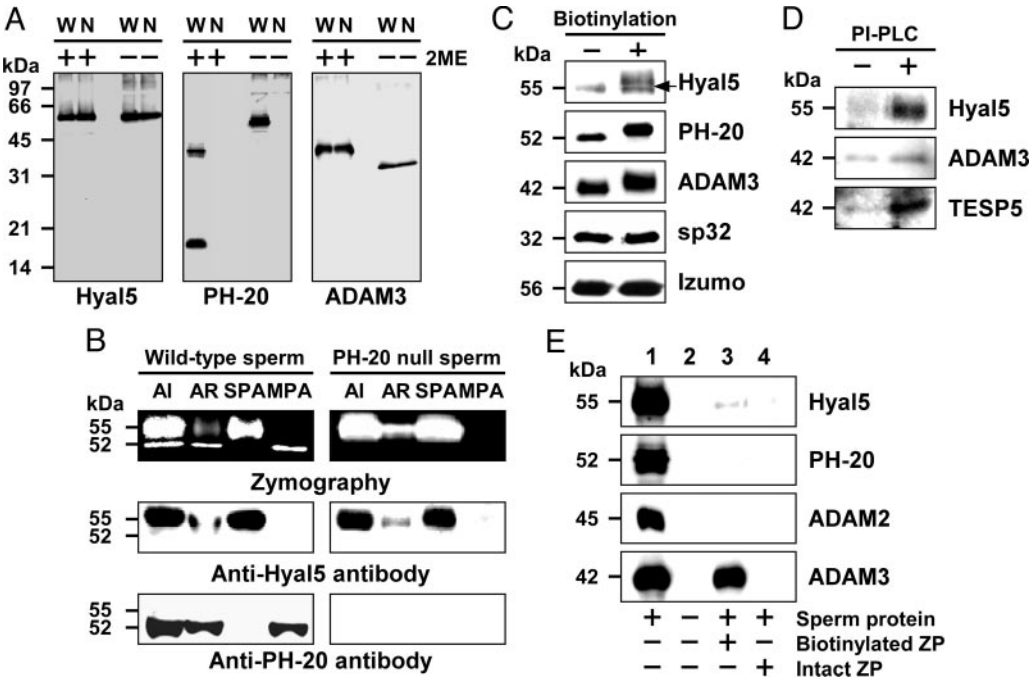

Fig. 3. Characterization of mouse Hyal5. (*A*) Immunoblot analysis of AI sperm proteins. Protein extracts (3 μg each) of epididymal sperm from wild-type (W) and PH-20-deficient (N) mice were treated with SDS in the presence (+) or absence (−) of 2-mercaptoethanol (2ME), separated by SDS/PAGE, and subjected to immunoblot analysis using anti-Hyal5, anti-PH-20, and anti-ADAM3 antibodies. (*B*) Subcellular localization of Hyal5 in epididymal sperm. Four protein fractions (AI, AR, SPA, and MPA fractions) were prepared from AI and AR sperm of wild-type and PH-20 null mice. Proteins (3 μg each) were separated by SDS/PAGE under nonreducing conditions and analyzed by zymography and immunoblotting. (*C*) Biotinylation of surface proteins on wild-type mouse sperm. Biotinylated sperm proteins (3 μg each) were analyzed by immunoblotting using antibodies indicated. An arrow indicates the border between the biotin-labeled and unlabeled proteins. The biotin-labeled and unlabeled Hyal5 were estimated to be 62.1 ± 2.3% and 37.9 ± 2.3% (means ± SD, $n = 3$), respectively. (*D*) PI-PLC treatment of epididymal sperm. After incubation with (+) or without (−) bacterial PI-PLC, Hyal5 in soluble proteins liberated from 5×10^5 sperm per lane was analyzed by immunoblotting. Transmembrane and GPI-anchored sperm proteins, ADAM3 and TESP5, were examined as negative and positive controls, respectively. (*E*) Binding of sperm proteins to biotinylated ZP. Biotinylated mouse ZP was mixed with Triton X-100 extracts from wild-type mouse sperm, and ZP-binding proteins were analyzed by immunoblotting.

tested, with some exceptions, whereas expression of *Hyalp1*, *Ph-20*, and *Hyal5* was specific for the testis. Both 2.2- and 2.1-kb mRNAs transcribed from *Ph-20* and *Hyal5* were first detectable in the testes of 20-day-old mice, and mRNA levels progressively increased during testicular development (Fig. 2*C*). Moreover, immunoblot analysis revealed that Hyal5, as well as ADAM2 (10) and TESP5 (21), is absent in epididymal luminal fluids and tissues (Fig. 2*D*), inconsistent with PH-20 (29). These data suggest that *Hyal5* is expressed exclusively in the testis, and the expression may be specific for pachytene spermatocytes and round spermatids. It should be noted that no DNA fragment corresponding to Hyal4 was amplified by RT-PCR (data not shown), consistent with the fact that *Hyal4* is expressed specifically in the placenta and skeletal muscle (5).

Immunoblot analysis of protein extracts from AI epididymal sperm (AI fraction) indicated the presence of 55-kDa Hyal5 in wild-type and $Ph20^{-/-}$ mice (Fig. 3*A*). The molecular size of Hyal5 was identical under nonreducing and reducing conditions, whereas 52-kDa PH-20 in the AI fraction of wild-type mice was separated into two polypeptides with the sizes of 43 and 18 kDa only under reducing conditions. These results demonstrate that Hyal5 is a single-chain molecule structurally distinguishable from PH-20 consisting of two chains covalently linked by one of two preexisting disulfide bridges (8, 17, 18, 30). Indeed, the endoproteolytic cleavage site sequences in guinea pig and mouse PH-20, Arg^{346}-Ser^{347}, and Arg^{347}-Ala^{348}, respectively, are replaced by Thr^{347}-Met^{348} in Hyal5 (Figs. 1*B* and 6).

To verify the subcellular localization of Hyal5 on epididymal sperm, AI, AR, SPA, and MPA fractions were prepared from AI and AR sperm (19) and analyzed by zymography and immunoblotting (Fig. 3*B*). The SPA fractions from wild-type and $Ph20^{-/-}$ mice contained only 55-kDa Hyal5, whereas 52-kDa PH-20 was present in the MPA fraction from wild-type mice, as described (19). We next asked whether Hyal5 is localized on the surface of AI epididymal sperm (Fig. 3*C*). Biotinylation of surface proteins on wild-type mouse sperm resulted in slow migration of PH-20 and ADAM3 on SDS/PAGE, whereas both sp32 and Izumo, present in the sperm acrosome (31) and on the cell surface after acrosome reaction (32), respectively, were not biotinylated. Unexpectedly, more than half of Hyal5 (≈62%) were only biotinylated, and the remainder (38%) was not. Moreover, Hyal5 as well as GPI-anchored serine protease TESP5 (21) was released from the sperm surface by PI-PLC treatment (Fig. 3*D*). Thus, mouse sperm possess GPI-anchored Hyal5 on the plasma membrane and retain it on the acrosomal membrane until acrosome reaction. Even after the acrosome reaction, PH-20 is still present on the sperm surface.

PH-20 is known to contain a functional domain to bind egg ZP (8, 9, 18), although $Ph20^{-/-}$ mouse sperm normally bind to egg ZP (19). To examine whether Hyal5 exhibits ZP-binding activity, binding assays were carried out by using biotinylated mouse ZP (Fig. 3*E*). Only ADAM3 among four proteins examined was capable of binding to the ZP tightly, as expected (24, 33). Hyal5 also bound to the ZP, but the level of ZP binding was negligibly low. Both PH-20 and ADAM2 apparently exhibited no binding activity under the conditions used. However, a very slightly immunoreactive band of PH-20 was occasionally detectable when the blots were overexposed (data not shown). These data may weaken the possibility that the ZP-binding domains of Hyal5 and PH-20 function in the sperm–egg interaction.

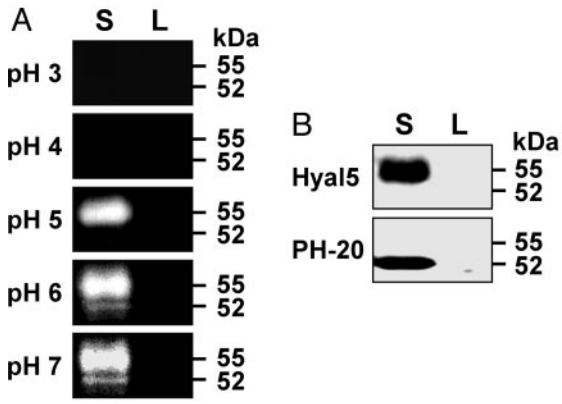

Fig. 4. pH dependence of Hyal5 and PH-20 activities. Soluble proteins (3 and 10 μg per lane) from mouse epididymal sperm (S) and liver (L), respectively, were separated by SDS/PAGE under nonreducing conditions and analyzed by hyaluronan zymography (*A*) and immunoblotting (*B*).

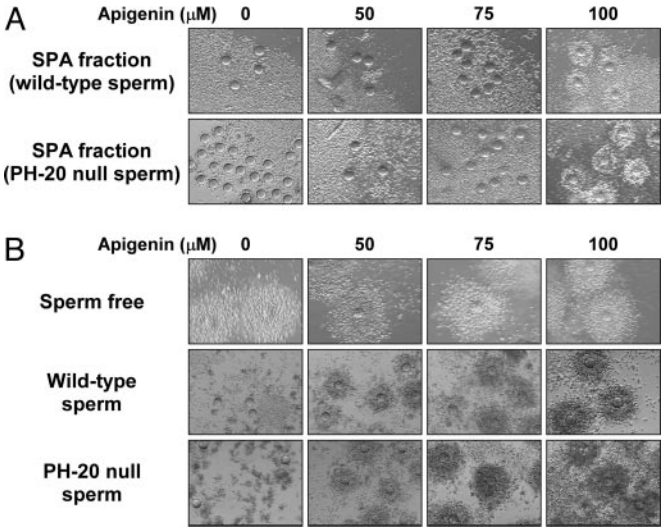

Fig. 5. Dispersal of cumulus cells from cumulus mass. (*A*) Cumulus cell dispersal by SPA fractions from wild-type and PH-20 null mouse sperm. The cumulus masses were incubated for 30 min with the SPA fractions in the absence or presence of a hyaluronidase inhibitor, apigenin, at the concentrations indicated. (*B*) Cumulus cell dispersal by wild-type and PH-20 null mouse sperm. The cumulus masses were inseminated by capacitated epididymal sperm of wild-type and PH-20 null mice in the absence or presence of apigenin, and the mixture was incubated for 3 h.

It has been reported that two forms of hyaluronidase are present in sperm of several mammalian species: PH-20 GPI-anchored on the plasma and inner acrosomal membranes and a soluble enzyme (sPH-20) released during acrosome reaction (17, 34, 35). In cynomolgus macaque, the GPI-anchored form exhibits the maximum activity at neutral pH, whereas sPH-20 is active only at acid pH (34, 35). As shown in Fig. 4, hyaluronan zymography revealed that mouse Hyal5 is active in the pH range 5–7 and inactive at pH 3 and 4. Mouse PH-20 activity was detectable only at pH 6 and 7. Thus, the pH dependencies of the mouse Hyal5 and PH-20 activities are similar to each other but distinguished from that of macaque sPH-20.

To ascertain the involvement of Hyal5 in the process of sperm penetration through the cumulus mass, *in vitro* fertilization assays were performed by using SPA protein fractions and epididymal sperm from wild-type and *Ph20*$^{-/-}$ mice. Despite the absence of PH-20, the two SPA fractions dispersed cumulus cells from the cumulus mass (Fig. 5*A*). Cumulus cell dispersal was strongly inhibited by the presence of a hyaluronidase inhibitor, apigenin (36), at 100 μM. The similar pattern of the cumulus cell dispersal was observed when bovine testicular hyaluronidase or partially purified preparation of Hyal5 (fraction 18 in Fig. 1*A*) was incubated with the cumulus mass (data not shown). Moreover, cumulus cells were dispersed by wild-type and *Ph20*$^{-/-}$ mouse sperm in the absence of apigenin (Fig. 5*B*). The dispersal was inhibited by apigenin in a concentration-dependent manner over the range of 50–100 μM. Thus, Hyal5 may be responsible for sperm penetration through the cumulus mass, although the participation of PH-20 cannot be ruled out completely. It is important to note that spontaneous acrosome reaction of epididymal sperm occurred during cumulus cell dispersal (17.8 $\pm$ 2.9% and 19.4 $\pm$ 3.8% in wild-type and *Ph20*$^{-/-}$ mouse sperm during the 3-h incubation with cumulus mass, respectively). Because the sperm acrosome contains $\approx$38% of total sperm Hyal5 (Fig. 3), a part of Hyal5 (almost 7% of total) is released by the spontaneous acrosome reaction and may contribute to the cumulus cell dispersal.

Discussion

This study describes the identification of a hyaluronidase, Hyal5, presumably involved in sperm penetration through cumulus mass in the mouse. Despite the similarities in the amino acid sequence, chromosomal localization, and gene expression pattern (Figs. 1, 2, and 6), Hyal5 is distinguished from PH-20 in several respects: the abundance, subcellular localization, and endoproteolytic processing of these two proteins on epididymal sperm (Fig. 3). Moreover, most or all Hyal5 is released from epididymal sperm during the acrosome reaction, although AR sperm still contain PH-20 (Fig. 3*B*). Protein synthesis in the epididymis is also distinctive in that it contains PH-20 (Fig. 2*D*).

Mouse epididymal sperm apparently contains only two hyaluronan-hydrolyzing proteins, Hyal5 and PH-20 (Fig. 3*B*). Because the hyaluronan-hydrolyzing activity band of Hyal5 is very broad as compared with that of PH-20, it is possible that other hyaluronidases comigrate to the same position with Hyal5 on SDS/PAGE. Indeed, five hyaluronidase genes, including *Hyal2*, *Hyal3*, and *Hyalp1*, are expressed in the mouse testis (Fig. 2*B*). When immunoblot analysis of protein extracts from AI sperm was carried out by using anti-mouse Hyalp1 antibody, no immunoreactive protein was found (data not shown). Although Hyal2 is known to exhibit the maximum activity at pH 4 (37), no hyaluronan-hydrolyzing activity was detected at pH 3 and 4 (Fig. 4). The calculated molecular mass of mouse Hyal3 was >12 kDa smaller than those of Hyal5 and PH-20. In addition, no peptide fragment derived from Hyal2, Hyal3, and Hyalp1 was identified in the purified Hyal5 preparation by peptide MS analysis (Fig. 1*A*). Thus, we conclude that mouse epididymal sperm contain only Hyal5 and PH-20. The broad activity band of Hyal5 may reflect the difference of the substrate specificity toward human umbilical cord hyaluronan between Hyal5 and PH-20, because these two proteins appear to be present equally on AI sperm.

In the guinea pig, PH-20 is endoproteolytically processed into two disulfide-linked polypeptides, whereas sPH-20 is not cleaved during the acrosome reaction (8, 17, 18). sPH-20, however, is indistinguishable from PH-20 by both immunoreactivity with mono- and polyclonal antibodies against PH-20 and molecular size on SDS/PAGE. Thus, sPH-20 is believed to be the released form of PH-20 from the plasma and/or inner acrosomal membranes during the acrosome reaction (17). Our data apparently imply that mouse Hyal5 may correspond to guinea pig sPH-20, because most or all single-chain Hyal5 is released into the SPA fraction by the acrosome reaction (Fig. 3*B*). However, mouse PH-20 and Hyal5 are the structurally distinct forms of sperm hyaluronidase (Figs. 1 and 6), thus indicating that Hyal5 is not identical to sPH-20 derived from PH-20. Rather, guinea pig sperm as well as mouse sperm may

possess two hyaluronidases corresponding to Hyal5 and PH-20. If so, sPH-20 and Hyal5 are probably the same in the guinea pig. Indeed, Northern blot analysis reveals the presence of Hyal5 mRNA in the testicular tissues from mouse, rat, hamster, and possibly rabbit and guinea pig (Fig. 8, which is published as supporting information on the PNAS web site).

Our data led us to evaluate the localization and function of Hyal5 and PH-20 on mouse sperm, although the anti-Hyal5 and anti-PH-20 antibodies used in this study are inapplicable to immunohistochemical analysis of sperm. As shown in Fig. 3*C*, PH-20 is all labeled with sulfo-NHS-LC-biotin, demonstrating that this hyaluronidase is localized only on the plasma membrane of AI sperm. Approximately half of PH-20 are retained on the plasma membrane (Fig. 3*B*) or possibly moved onto the inner acrosomal membrane (16, 38) after the acrosome reaction. The remainder of PH-20 is still on the vesicles derived from the plasma and outer acrosomal membranes fused during the acrosome reaction (MPA fraction), and then the vesicles are dispersed from sperm or adhere to the sperm head (Fig. 3*B*). In the case of Hyal5, this protein is present on the plasma and acrosomal membranes of AI sperm and released during the acrosome reaction (Fig. 3 *B* and *C*). Despite that the precise localization of Hyal5 on the acrosomal membrane is unclear at present, at least AR sperm barely contain Hyal5 on the inner acrosomal membrane (Fig. 3*B*). Thus, hyaluronidase activity of Hyal5 on the sperm surface at neutral pH (Figs. 3 and 4) is likely responsible for the sperm penetration through cumulus mass in the mouse. Both the reserve of Hyal5 on the acrosomal membrane and the release of this enzyme by the acrosome reaction suggest that Hyal5 may function also as a cumulus matrix depolymerase near or on the surface of ZP locally to enable the proximal region of the sperm tail to move more freely, according to a hypothetical model (39, 40). Indeed, Hyal5 is enzymatically active at pH 5 and 6 (Fig. 4), which is consistent with the fact that the intraacrosomal pH values of mouse sperm are estimated to be 5.3 and 6.2 immediately after sperm preparation and 2 h after capacitation in TYH medium, respectively (41). PH-20 seems to compensate in part for the functional roles of Hyal5.

As mentioned above, three genes encoding HYALP1, HYAL4, and PH-20/SPAM1 are known to form a cluster on the human chromosome 7 (5, 6). Our results show that a 160-kbp gene cluster on mouse chromosome 6 includes *Hyal5*, in addition to these three genes (Fig. 2*A*). A computer-aided BLAST search for the ortholog of mouse *Hyal5* on the National Center for Biotechnology Information database (www.ncbi.nih.gov/BLAST) reveals the presence of *Hyal5* on rat chromosome and the absence of the orthologs on the human, pig, cow, and chimpanzee chromosomes (Fig. 9, which is published as supporting information on the PNAS web site). Rat *Hyal5* is localized 28 kbp away from *Ph-20* on chromosome 4, and rat Hyal5 as the gene product shares a high degree of sequence identity (83%) with mouse Hyal5 (55% with rat PH-20). Importantly, the genomic region carrying *Hyal5* (≈60–90 kbp) is inserted only into the mouse and rat chromosomes (Fig. 9). It is thus conceivable that *Hyal5* is present exclusively in some rodents and guinea pig (Fig. 8). If so, PH-20 may still be essential for sperm penetration through the cumulus mass in the world of the other animals lacking *Hyal5* on the chromosomes.

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1. Laurent, T. C. & Fraser, J. R. E. (1992) *FASEB J.* **6,** 2397–2404.
2. Kreil, G. (1995) *Protein Sci.* **4,** 1666–1669.
3. Frost, G. I., Csoka, T. & Stern, R. (1996) *Trends Glycosci. Glycotechnol.* **8,** 419–434.
4. Meyer, K. (1971) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), 3rd Ed., Vol. 5, pp. 307–320.
5. Csóka, A. B., Scherer, S. W. & Stern, R. (1999) *Genomics* **60,** 356–361.
6. Csóka, A. B., Frost, G. I. & Stern, R. (2001) *Matrix Biol.* **20,** 499–508.
7. Yanagimachi, R. (1994) in *The Physiology of Reproduction*, eds, Knobil, E. & Neill, J. D. (Raven, New York), pp. 189–317.
8. Myles, D. G. & Primakoff, P. (1997) *Biol. Reprod.* **56,** 320–327.
9. Cherr, G. N., Yudin, A. I. & Overstreet, J. W. (2001) *Matrix Biol.* **20,** 515–525.
10. Primakoff, P. & Myles, D. G. (2002) *Science* **296,** 2183–2185.
11. Dandekar, P., Aggeler, J. & Talbot, P. (1992) *Hum. Reprod.* **7,** 391–398.
12. Phelps, B. M., Primakoff, P., Koppel, D. E., Low, M. G. & Myles, D. G. (1988) *Science* **240,** 1780–1782.
13. Gmachl, M. & Kreil, G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3569–3573.
14. Lin, Y., Kimmel, L. H., Myles, D. G. & Primakoff, P. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10071–10075.
15. Lin, Y., Mahan, K., Lathrop, W. F., Myles, D. G. & Primakoff, P. (1994) *J. Cell Biol.* **125,** 1157–1163.
16. Overstreet, J. W., Lin, Y., Yudin, A. I., Meyers, S. A., Primakoff, P., Myles, D. G., Katz, D. F. & VandeVoort, C. A. (1995) *Biol. Reprod.* **52,** 105–114.
17. Hunnicut, G. R., Mahan, K., Lathrop, W. F., Ramarao, C. S., Myles, D. G. & Primakoff, P. (1996) *Biol. Reprod.* **54,** 1343–1349.
18. Hunnicutt, G. R., Primakoff, P. & Myles, D. G. (1996) *Biol. Reprod.* **55,** 80–86.
19. Baba, D., Kashiwabara, S., Honda, A., Yamagata, K., Wu, Q., Ikawa, M., Okabe, M. & Baba, T. (2002) *J. Biol. Chem.* **277,** 30310–30314.
20. Toyoda, Y., Yokoyama, M. & Hoshi, T. (1971) *Jpn. J. Anim. Reprod.* **16,** 147–151.
21. Honda, A., Yamagata, K., Sugiura, S., Watanabe, K. & Baba, T. (2002) *J. Biol. Chem.* **277,** 16976–16984.
22. Yamagata, K., Honda, A., Kashiwabara, S. & Baba, T. (1999) *Dev. Genet.* **25,** 115–122.
23. Guntenhöner, M. W., Pogrel, M. A. & Stern, R. (1992) *Matrix* **12,** 388–396.
24. Nishimura, H., Kim, E., Nakanishi, T. & Baba, T. (2004) *J. Biol. Chem.* **279,** 34957–34962.
25. Kim, E., Nishimura, H. & Baba, T. (2003) *Biochem. Biophys. Res. Commun.* **304,** 313–319.
26. Kim, E., Nishimura, H., Iwase, S., Yamagata, K., Kashiwabara, S. & Baba, T. (2004) *J. Reprod. Dev.* **50,** 571–578.
27. Yamagata, K., Murayama, K., Okabe, M., Toshimori, K., Nakanishi, T., Kashiwabara, S. & Baba, T. (1998) *J. Biol. Chem.* **273,** 10470–10474.
28. Zhang, H., Shertok, S., Miller, K., Taylor, L. & Martin-DeLeon, P. A. (2005) *Mol. Reprod. Dev.* **72,** 404–410.
29. Zhang, H. & Martin-DeLeon, P. A. (2001) *Biol. Reprod.* **65,** 1586–1593.
30. Marković-Housley, Z., Miglierini, G., Soldatova, L., Rizkallah, P. J., Müller, U. & Schirmer, T. (2000) *Structure (Cambridge, U.K.)* **8,** 1025–1035.
31. Baba, T., Niida, Y., Michikawa, Y., Kashiwabara, S., Kodaira, K., Takenaka, M., Kohno, N., Gerton, G. L. & Arai, Y. (1994) *J. Biol. Chem.* **269,** 10133–10140.
32. Inoue, N., Ikawa, M., Isotani, A. & Okabe, M. (2005) *Nature* **434,** 234–238.
33. Shamsadin, R., Adham, I. M., Nayernia, K., Heinlein, U. A. O., Oberwinkler, H. & Engel, W. (1999) *Biol. Reprod.* **61,** 1445–1451.
34. Cherr, G. N., Meyers, S. A., Yudin, A. I., VandeVoort, C. A., Myles, D. G., Primakoff, P. & Overstreet, J. W. (1996) *Dev. Biol.* **175,** 142–153.
35. Li, M. -W., Cherr, G. N. Yudin, A. I. & Overstreet, J. W. (1997) *Mol. Reprod. Dev.* **48,** 356–366.
36. Li, M. -W., Yudin, A. I., VandeVoort, C. A., Sabeur, K., Primakoff, P. & Overstreet, J. W. (1997) *Biol. Reprod.* **56,** 1383–1389.
37. Lepperdinger, G., Müllegger, J. & Kreil, G. (2001) *Matrix Biol.* **20,** 509–514.
38. Phelps, B. M., Koppel, D. E., Primakoff, P. & Myles, D. G. (1990) *J. Cell Biol.* **111,** 1839–1847.
39. Drobnis, E. Z., Yudin, A. I., Cherr, G. N. & Katz, D. F. (1988) *Gamete Res.* **21,** 367–383.
40. Drobnis, E. Z., Yudin, A. I., Cherr, G. N. & Katz, D. F. (1988) *Dev. Biol.* **130,** 311–323.
41. Nakanishi, T., Ikawa, M., Yamada, S., Toshimori, K. & Okabe, M. (2001) *Dev. Biol.* **237,** 222–231.