

A Hyaluronidase Activity of the Sperm Plasma Membrane Protein PH-20 Enables Sperm to Penetrate the Cumulus Cell Layer Surrounding the Egg

Ying Lin, Kimberly Mahan, William F. Lathrop, Diana G. Myles, and Paul Primakoff

Department of Physiology, University of Connecticut Health Center, Farmington, Connecticut 06030

Abstract. A typical mammalian egg is surrounded by an outer layer of about 3,000 cumulus cells embedded in an extracellular matrix rich in hyaluronic acid. A current, widely proposed model is that the fertilizing sperm, while it is acrosome intact, passes through the cumulus cell layer and binds to the egg zona pellucida. This current model lacks a well-supported explanation for how sperm penetrate the cumulus layer. We report that the sperm protein PH-20 has a hyaluronidase activity and is present on the plasma membrane

of mouse and human sperm. Brief treatment with purified, recombinant PH-20 can release all the cumulus cells surrounding mouse eggs. Acrosome intact mouse sperm incubated with anti-PH-20 antibodies can not pass through the cumulus layer and thus can not reach the zona pellucida. These results, indicating that PH-20 enables acrosome intact sperm to penetrate the cumulus barrier, reveal a mechanism for cumulus penetration, and thus provide the missing element in the current model.

FOR fertilization to occur the ability of sperm to reach the egg is as critical as the ensuing events of sperm-egg adhesion and fusion. In mammals a collection of physiological processes including sperm motility, ciliary action in the oviduct, and muscular activity in the vaginal, cervical, and oviductal walls allow the sperm and egg to approach each other in the female reproductive tract (10). After the gametes approach each other, the sperm must have a means to penetrate the structures surrounding the egg in order to reach, and ultimately fuse with, the egg plasma membrane.

A single ovulated mammalian (mouse) egg is surrounded by about 3,000 cumulus cells. Before ovulation the spacing between cumulus cells increases as the result of synthesis of increased extracellular matrix (ECM) components, including hyaluronic acid (HA). This process, termed cumulus expansion, is regulated by gonadotropins and soluble factor(s) from the oocyte (31, 32). Expansion results in a cumulus cell/ECM layer that presents a potentially formidable obstacle to an approaching sperm cell.

Various ideas of how sperm may penetrate the cumulus layer have been proposed by different investigators. In an

early model, sperm were believed to acrosome react outside the cumulus releasing their soluble, acrosomal contents including hyaluronidase. The soluble hyaluronidase was thought to yield sufficient HA cleavage in the ECM of the cumulus cells to allow the acrosome-reacted sperm to penetrate the cumulus layer and proceed to fertilize the egg (20, 34, 41).

This early model proved to be inconsistent with more recent investigations that examined the acrosomal status of sperm passing through the cumulus. It was found that sperm that have completed their acrosome reaction before meeting the cumulus stick to the surface of the cumulus and cannot penetrate (5, 7, 35, 37, 41). Furthermore, in both hamster and mouse *in vitro* fertilization studies, investigators found that it is acrosome intact sperm that penetrate the cumulus layer and reach the zona (1, 3, 7, 13, 34, 41). Consistent with these findings was the demonstration in several species that the zona, in particular ZP-3, induces sperm to acrosome react (reviewed in 1, 13, 41). These results have led to a widely proposed, current model which states that a fertilizing, acrosome intact sperm passes through the cumulus layer, binds to the zona pellucida and is then induced to acrosome react (1, 3, 7, 13, 21, 34, 39–41). This model, as it is usually stated, lacks an explanation for how acrosome intact sperm penetrate the cumulus (1, 41).

Certain attempts have been made to explain how acrosome intact sperm might pass through the cumulus and reach the zona. Some investigators posited that enzyme activity (in particular hyaluronidase activity) is unnecessary for motile cells to penetrate the cumulus layer. Talbot et al. (38) reported that *Chlamydomonas* and sea urchin and frog sperm

Address all correspondence to Y. Lin, Department of Physiology, University of Connecticut Health Center, Farmington, CT 06030.

1. *Abbreviations used in this paper:* CNBr, cyanogen bromide; cPH-20, cynomolgus monkey; ECM, extracellular matrix; GPI, glycosyl phosphatidylinositol; gpPH-20, guinea pig PH-20; HA, hyaluronic acid; hPH-20, human PH-20; mPH-20, mouse PH-20; mT, modified Tyrode's medium; PI-PLC, phosphatidylinositol-specific phospholipase C; R-123, rhodamine-123.

showed no hyaluronidase activity, yet they could reach the zona of cumulus-intact hamster eggs. The interpretation of their experiments has been questioned (7) in that artifactual channels may have been present in the hamster cumulus layers, particularly under the ionic conditions used to maintain heterologous sperm motility. Other investigators have posited that many nonfertilizing, mammalian sperm acrosome react "spontaneously" outside the cumulus, releasing soluble hyaluronidase and thus "softening up" the cumulus for the passage of the one fertilizing, acrosome intact sperm (41). This "sperm suicide wave" theory is inconsistent with data showing that in vivo in mammals only one, or a few, sperm are present at the site of fertilization (41).

The investigation in this report arose initially from a very different direction than the issue of sperm penetration of the cumulus layer. Our lab has been studying the sperm surface protein PH-20 which has a required function in a later step in fertilization, when acrosome-reacted sperm bind to the zona pellucida (23, 27, 28). Some years ago we cloned and sequenced guinea pig PH-20 (gpPH-20) cDNA (15). Recently, Gmachl and Kreil (9) isolated a cDNA for bee venom hyaluronidase and reported that it had homology with an NH₂-terminal domain of gpPH-20 (the NH₂-terminal 307 residues). This finding prompted us to ask if PH-20 might have a hyaluronidase activity and what role such an activity could have in the process of fertilization.

Materials and Methods

PCR Cloning of Mouse PH-20-KT3 and Cynomolgus Monkey PH-20-KT3

PCR was used to synthesize fragments of mouse PH-20 (mPH-20) and cynomolgus monkey (cPH-20) cDNAs truncated at the 3' ends. The fragments were truncated to encode PH-20 lacking the signal for attachment of the GPI membrane anchor, so that the encoded PH-20 would be secreted. In addition the engineered PH-20 carried the KT3 epitope tag: the 3' PCR primers contained, before the translation stop, a sequence encoding a 6-amino acid epitope tag (Pro-Pro-Glu-Pro-Glu-Thr) for mPH-20 or an 8-amino acid tag (Thr-Pro-Pro-Pro-Glu-Pro-Glu-Thr) for cPH-20. Both tags are recognized by the mAb KT3. PCR primers were as follows: mouse 5' primer = 5' GCGAATTCGCTAGCTGTGATGGAGAGTTGAG-ATTAAAG 3' and cynomolgus 5' primer = 5' GCGGGATCCGCTAGCTTTACAATGGGAGTGC 3'; mouse 3' primer = 5' GCGAATTCGCTAGCTAGGTTCTCAGGCTCAGGAGGTAGGAGGTAGAAGGCTGG 3' and cynomolgus 3' primer = 5' GCGGGATCCGCTAGCTCAIGTTCTGGTTCTGGTGGTGGTGTCTCTCCTGTCTCCAATTGG 3' (nucleotides encoding the KT3 epitope are underlined). PCR conditions were as described (18). The products obtained were subcloned into the NheI or BamHI site of the baculovirus transfer vector pJVP10Z (a gift from Dr. Chris Richardson, McGill University, Montreal).

Transfection of Sf-9 Cells and Production of Recombinant Baculovirus

Sequences encoding mPH-20-KT3 and cPH-20-KT3 were recombined into the Autographa californica baculovirus (AcNPV) via the pJVP10Z transfer vector containing PH-20-KT3 inserts. To generate recombinant virus, 1 µg of transfer vector was cotransfected with 1 µg of wild-type viral DNA into Sf-9 cells as described (36). Recombinant viruses were isolated from transfection supernatants by plaque purification. To produce recombinant protein, Sf-9 cells were infected with 0.1 PFU of recombinant virus per cell. Suspension cultures were grown in a stirred flask containing serum-free medium. The cultures were infected at 2×10^6 cells/ml and were harvested at 4-d postinfection. The expression level of recombinant, secreted protein was ~1 mg/liter of Sf-9 culture.

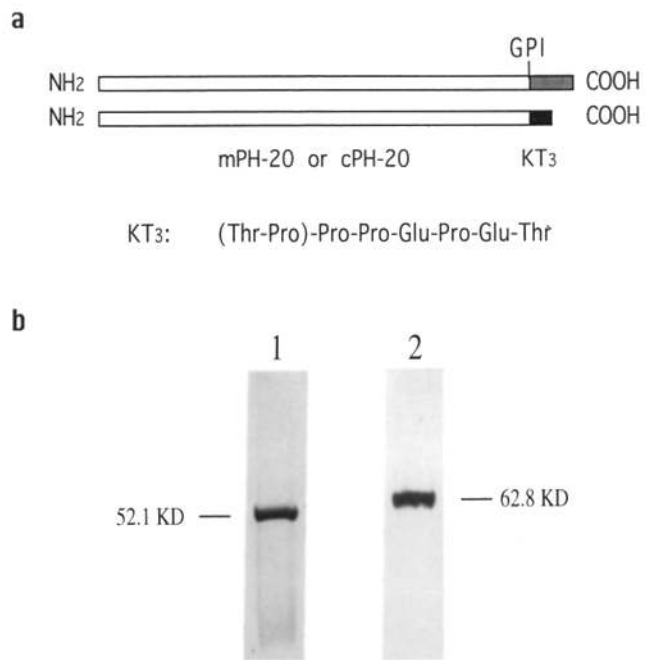


Figure 1. Expression of recombinant PH-20 in insect cells. (a) Fragments of mPH-20 and cPH-20 cDNA corresponding to the coding region without the GPI anchor at the COOH-terminus were synthesized by PCR. The 3' PCR primers contained before the translation stop a sequence encoding a 6-amino acid epitope tag (Pro-Pro-Glu-Pro-Glu-Thr) for mPH-20 or an 8-amino acid tag (Thr-Pro-Pro-Pro-Glu-Pro-Glu-Thr) for cPH-20, both recognized by the mAb KT3. Recombinant, secreted PH-20 was expressed in Sf9 cells and affinity purified on a KT3-Sepharose column. (b) Silver-stained SDS-PAGE of purified, recombinant mPH-20 (lane 1), and cPH-20 (lane 2). SDS-PAGE analysis using silver staining was done on 5.0 µg purified mPH-20 (relatively short stain development) and 1.0 µg cPH-20 (relatively long stain development).

Purification of Recombinant Protein

KT3-Sepharose was prepared by coupling KT3 mAb (19) to cyanogen bromide (CNBr)-activated Sepharose CL-4B (7.8 mg KT3/ml resin). Culture media from infected cells were concentrated 10× and loaded on the KT3-Sepharose column at 4°C at a flow rate of 2 bed vol/h. The column was then washed with 5 vol of TNE (10 mM Tris, 140 mM NaCl, 5 mM EDTA, pH 8.0) and eluted with 0.1 M glycine (pH 3.0). Eluted fractions were neutralized with 1 M Tris (pH 9.0) and dialyzed against 2 liters of PBS.

Production of Polyclonal Antibodies

Polyclonal antisera were generated by injecting a female New Zealand White rabbit with 100 µg purified recombinant mPH-20-KT3 or cPH-20-KT3 in complete Freund's adjuvant. Rabbits were boosted twice, 3-wk apart, with the same dose of antigen in incomplete Freund's adjuvant. Sera were collected 2-wk after the second boost by heart puncture.

Hyaluronidase Activity Assay

Enzyme assays were performed according to Dorfman (6). Briefly, recombinant PH-20 protein was dissolved in 1 ml enzyme buffer (0.02 M phosphate buffer, 0.45% NaCl, 0.01% BSA, pH 6.8-7.0) and mixed with 0.5 mg HA which was dissolved in 1 ml substrate buffer (0.3 M KH₂PO₄/Na₂HPO₄, pH 5.30-5.35). Enzyme digestion was allowed to proceed for 45 min at 37°C. Turbidity was generated at the end of incubation by adding 10 ml acid albumin solution. Optical density at 600 nm was determined exactly 5 min after addition of acid albumin. A standard testicular hyaluronidase (H-3506; Sigma Chemical Co., St. Louis, MO) containing 0.29 U/µg was used to

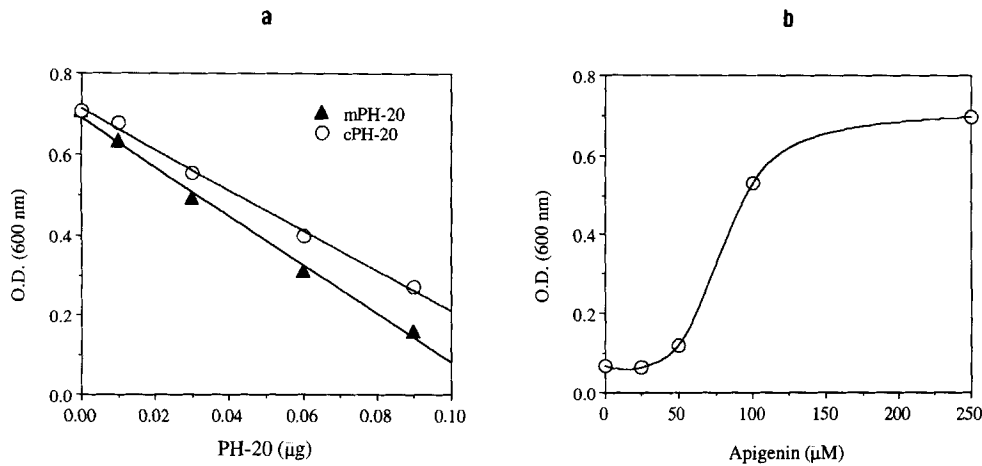


Figure 2. Assay of the hyaluronidase activity of purified, recombinant PH-20 proteins. (a) Enzyme activity of mPH-20 and cPH-20 was determined according to Dorfman (6). (b) Inhibition of PH-20 enzyme activity by the hyaluronidase inhibitor apigenin. 0.5 mg cPH-20 was first incubated with apigenin at various concentrations at room temperature for 20 min and then assayed for enzyme activity.

establish a standard curve and units of activity were calculated by comparison to this curve.

Indirect Immunofluorescence on Mouse and Human Sperm

For mouse sperm, fresh cauda epididymal sperm were collected into Whittingham's medium (mT) (11) and for human sperm, freshly ejaculated motile sperm were selected on a Percoll gradient (33) and washed through mT. Sperm were then incubated at room temperature for 60 min with either preimmune sera (diluted 1:100) or immune anti-cPH-20 serum (diluted 1:100) raised against purified, recombinant cPH-20-KT3. After washing through mT containing 3% BSA and incubating with rhodamine-conjugated goat anti-rabbit Fab at room temperature for 60 min, sperm were fixed in 1.5% paraformaldehyde, and then viewed with a Zeiss Axioptot microscope. Phase contrast and fluorescence images (780×) were photographed for the same cell.

Cleavage of PH-20 Protein from the Sperm Surface by Phosphatidylinositol-specific Phospholipase C

Freshly collected mouse cauda epididymal and human ejaculated sperm were washed through PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂, pH 6.9), evenly divided into two tubes containing either buffer or buffer plus 45 U phosphatidylinositol-specific phospholipase C (PI-PLC) (ICN Biomedicals, Irvine, CA), and then incubated for 30 min at 37°C. Sperm and particulates were pelleted by a low-speed centrifugation, followed by a high-speed centrifugation (100,000 g for 30 min). Supernatants from the 100,000 g spin derived from 5 × 10⁶ mouse sperm or 10⁷ human sperm were run on 4–15% SDS-polyacrylamide gels and Western blotted with polyclonal antibodies against recombinant PH-20.

Release of Cumulus Cells from Mouse Eggs

Superovulated mouse eggs, collected in Whittingham's medium (11) with

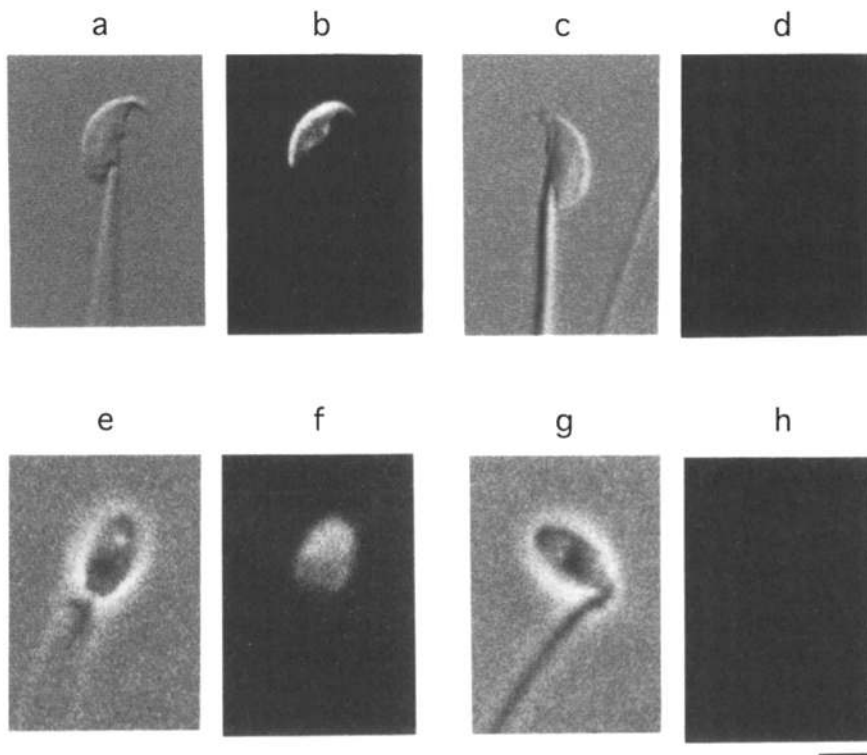


Figure 3. Immunofluorescence of live, acrosome intact mouse (a–d) and human (e–h) sperm. Live sperm were incubated with either anti-cPH-20 rabbit serum (a and b, e and f), raised against purified, recombinant cPH-20 or preimmune serum (c and d, g and h). Paired micrographs (a and b, c and d, e and f, g and h), are phase and fluorescence images of the same cell (780×). mPH-20 is on the anterior head surface (b) and hPH-20 is on the anterior or whole head surface (f). Bar, 10 μm.

0.3% BSA 60-h postinjection of PMSG and 12-h postinjection of hCG, were distributed into four dishes each containing 50 μ l medium under mineral oil. Eggs were then treated with different reagents for 5 min at room temperature. Treated eggs were washed and transferred to glass slides and photographed.

In Vitro Assay of Mouse Sperm Penetration through the Cumulus Layer of Mouse Eggs

Superovulated mouse eggs were collected as described above. Cauda epididymal mouse sperm in Whittingham's medium were capacitated at 37°C for 1 h, stained with 10 μ M rhodamine-123 (R-123) for 30 min, and washed through Whittingham's medium containing 10% BSA. Eggs were placed in 100- μ l insemination drops containing test antibodies in Whittingham's medium or medium alone. For the experiments illustrated in Fig. 6, "high" sperm concentration was used: 3×10^3 labeled sperm were added to each 100- μ l drop (containing five eggs) and incubated at 37°C for 10 min. For the experiments compiled in Table I, "low" sperm concentration was used: sperm, pre-incubated with 10 μ M R-123 and antibodies (1/50) for 30 min, were washed, and then 100–200 washed sperm were added to each 100- μ l insemination drop (containing \sim 10 eggs). Sperm and eggs were incubated together at 37°C for 10 min. In some cases, the 100- μ l insemination drop also contained a 1/50 dilution of the antibody as noted in Table I. Absence of antibody from the insemination drop may allow some antibody dissociation from the sperm, while presence of antibody in the insemination drop insures that essentially all antibody-binding sites remain occupied. Control antibodies were present in the insemination drop in all cases. After incubation eggs were fixed in 1% paraformaldehyde, mounted, and scored for sperm location (in relation to the cumulus layer) in one plane of focus near the center of the egg with a confocal microscope (Bio-Rad Laboratories, Cambridge, MA).

Results

Hyaluronidase Activity and Plasma Membrane Anchoring of PH-20

Our laboratory has previously cloned cDNAs for PH-20 from four mammalian species, initially guinea pig (15) and subsequently mouse (Lathrop, W. F., E. P. Carmichael, D. G. Myles, and P. Primakoff. 1990. *J. Cell Biol.* 115:462a), cynomolgus monkey, and human (18). gpPH-20 is 468-amino acids long. Bee venom hyaluronidase is 349-amino acids long and amino acids 14–303 have 36% identity to the NH₂-terminal amino acids 17–307 of gpPH-20. There is no homology in the COOH-terminal region of PH-20, i.e., residues 308–468 (9, 15).

To determine if PH-20 has hyaluronidase activity, we developed a system to express and purify recombinant PH-20. Constructs were made that code for secreted forms of mPH-20 and cPH-20 carrying the KT3 epitope tag. Recombinant baculoviruses containing these constructs were used to infect Sf9 insect cells (Fig. 1 a). Both secreted mPH-20 and cPH-20 were purified to homogeneity by affinity chromatography using the KT3 mAb coupled to Sepharose. The purified, recombinant proteins did not show detectable contamination on silver stained gels (Fig. 1 b).

To test for hyaluronidase activity in the purified, recombinant PH-20 we used the turbidimetric assay of Dorfman (6). We found that purified mPH-20 and cPH-20 have hyaluronidase activity, determined to be \sim 150 U/ μ g for mPH-20 and \sim 100 U/ μ g for cPH-20 (Fig. 2 a). In comparison, commercially available testicular hyaluronidase (Sigma Chemical Co.) has \sim 0.3–15 U/ μ g. The PH-20 enzyme activity is inhibited by apigenin, a flavonoid found to be an inhibitor of other hyaluronidases (14) (Fig. 2 b).

gpPH-20 has been previously found to be on the plasma membrane of guinea pig sperm (4, 22, 26), anchored in the

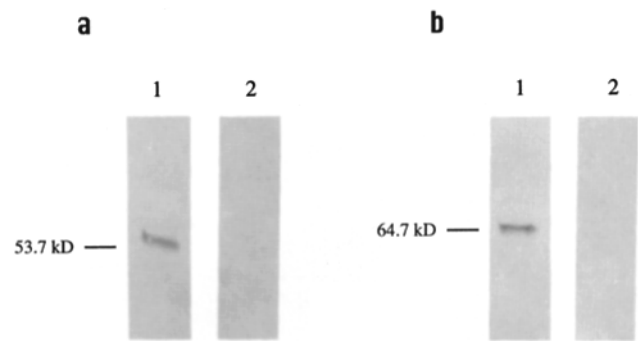


Figure 4. PH-20 is linked to the plasma membrane through a GPI anchor. Live mouse (a) and human (b) sperm incubated with PI-PLC released PH-20 (lanes 1). Control sperm, incubated without PI-PLC, showed no release (lanes 2).

lipid bilayer by a glycosyl phosphatidylinositol (GPI) anchor (24). In the current experiments, we found that PH-20 is also on the plasma membrane of mouse and human sperm. We stained live, acrosome intact mouse or human sperm with polyclonal antisera raised against purified, recombinant PH-20. Fluorescent staining was observed on the anterior head of mouse sperm on the anterior or whole head of human sperm (Fig. 3, a–h). To ask if mPH-20 and human PH-20 (hPH-20) are anchored in the plasma membrane by a GPI anchor, we treated live, acrosome intact sperm with PI-PLC. Incubation with PI-PLC released both mPH-20 and hPH-20 from the sperm plasma membrane (Fig. 4), indicating that PH-20 in both of these species has a GPI anchor, as it does in guinea pig.

Role of PH-20 Hyaluronidase Activity in Sperm Penetration of the Cumulus Layer

We hypothesized that the plasma membrane-anchored PH-20 hyaluronidase activity could digest HA outside cumulus cells and thus allow acrosome intact sperm penetration through the cumulus layer. To test this hypothesis we specifically chose to use mouse gametes because an extensive set of studies has shown that in mouse only acrosome intact sperm initiate binding to the zona of cumulus intact or cumulus free eggs (1, 2, 8, 17, 29, 30, 34, 40). To determine if PH-20 could have an effect on the mouse cumulus cell layer, we treated cumulus-intact mouse eggs with purified, recombinant PH-20. While untreated and buffer treated eggs kept their cumulus cells attached tightly for hours (Fig. 5, a and c), eggs treated with purified PH-20 lost their cumulus cells within 5 min (Fig. 5 b). When purified PH-20 was preincubated with an anti-PH-20 polyclonal antibody that inhibited its hyaluronidase activity, the cumulus cell releasing effect was blocked (Fig. 5 d).

We next tested if inhibition of PH-20's hyaluronidase activity on acrosome intact mouse sperm would block sperm penetration through the cumulus of mouse eggs. We inseminated cumulus intact mouse eggs with capacitated mouse sperm (\sim 3,000 sperm/100- μ l drop) after pre-incubating the sperm with a control antibody or an antibody blocking mPH-20 hyaluronidase activity. For a control antibody we used an anti-cPH-20 antibody, which cross-reacts with mPH-20 on mouse sperm (see Fig. 3 b), but does not inhibit mPH-20 hyaluronidase activity (data not shown). In the presence of the control antibody, numerous sperm could be observed that

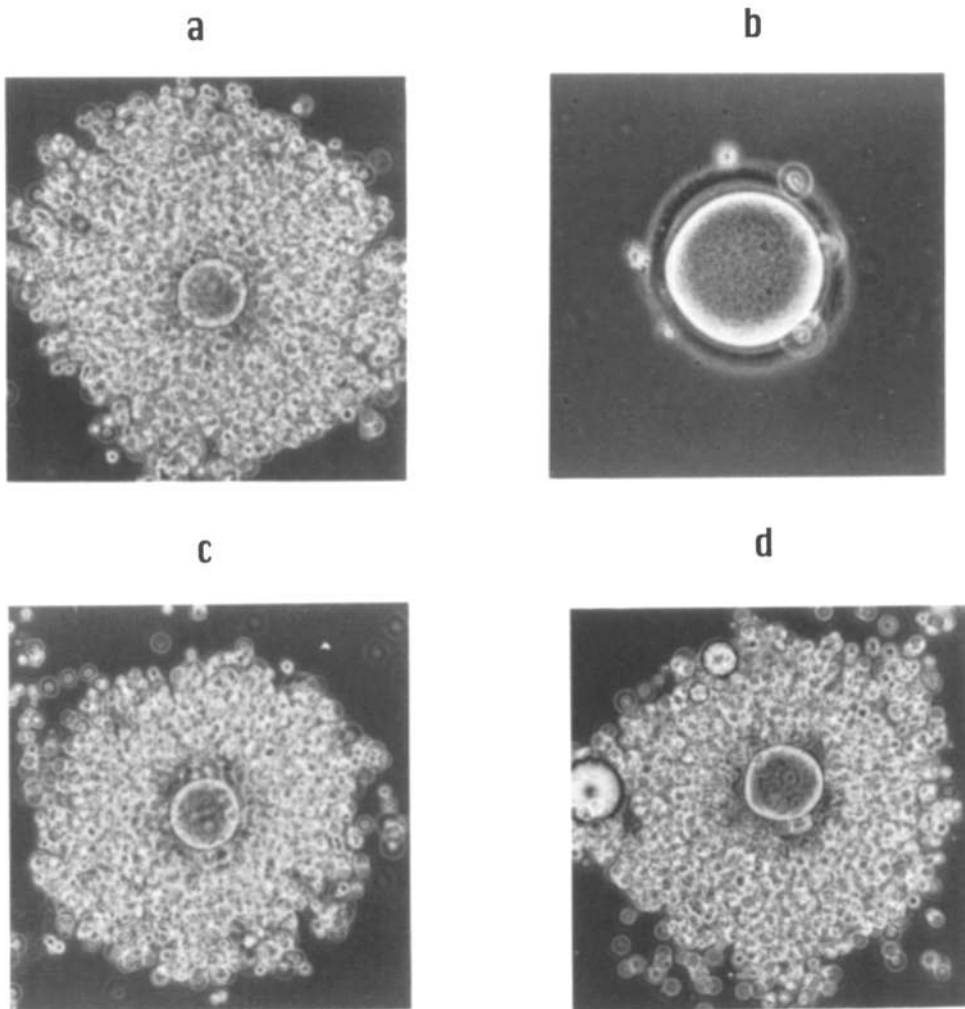


Figure 5. Purified, recombinant PH-20 releases cumulus cells from eggs. Cumulus intact mouse eggs were treated with either mPH-20 or cPH-20 with identical results. The eggs shown were incubated in PBS for 5 min at room temperature with these additions: (a) no addition, cumulus intact; (b) 5 μ g purified cPH-20 added, cumulus lost; (c) 5 μ g cPH-20 added in the presence of anti-cPH-20 antiserum (which inhibits cPH-20 hyaluronidase activity), cumulus intact; (d) buffer (PBS) added, cumulus intact.

penetrated the cumulus and reached the zona (Fig. 6 a). In contrast, when anti-mPH-20 that inhibits mPH-20 hyaluronidase activity was added, sperm remained outside, or only partially entered, the cumulus and no sperm reached the zona pellucida (Fig. 6 b).

Using cumulus-intact eggs inseminated with a much lower sperm concentration (\sim 100–200 sperm/100- μ l drop), we

could obtain quantitative data on sperm penetration through the cumulus. Under these conditions, a high percentage (86–100%) of the sperm reached the zona in controls (Table I). However, when PH-20 hyaluronidase activity on the mouse sperm was inhibited with the anti-mPH-20 antibody, almost no sperm reached the zona and 92–99% of the sperm remained outside the cumulus (Table I).

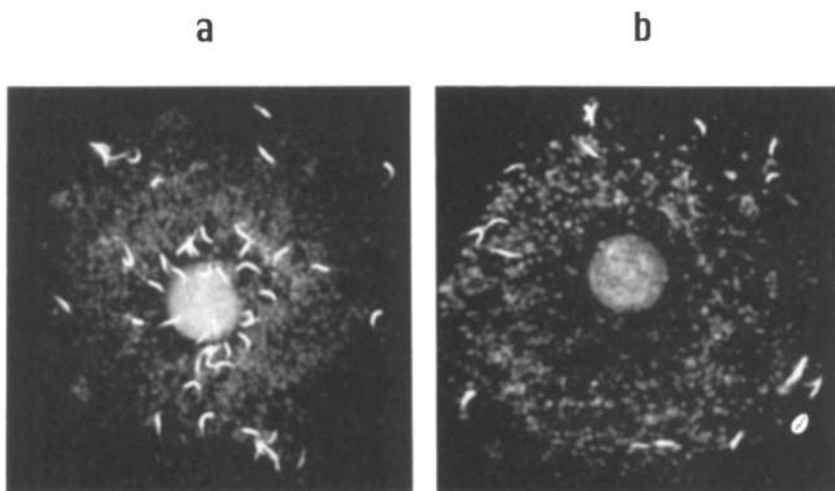


Figure 6. Anti-mPH-20 antibody prevents mouse sperm from penetrating the cumulus. Mouse sperm, labeled with R-123, were added to mouse eggs in the presence of different anti-PH-20 antibodies. (a) Sperm penetrate the cumulus and reach the zona in the presence of anti-cPH-20 antiserum which cross-reacts with mPH-20 (cf. Fig. 3 b) but does not inhibit mPH-20 hyaluronidase activity. (b) Sperm can not penetrate the cumulus layer in the presence of anti-mPH-20 antiserum which inhibits mPH-20 hyaluronidase activity. Sperm also penetrated the cumulus as in a when incubated with no addition or other control sera (pre-immune of the anti-mPH-20 and a rabbit antiserum to gpPH-30 (25) that binds to mPH-30).

Table I. Antibody Inhibition of Sperm Penetration through the Cumulus Layer

Sperm pre-incubated with:	Number of experiments	Number of eggs scored	Number of sperm observed	Number of sperm (Percent of total sperm)		
				Outside cumulus	Entered cumulus	Reached zona
No addition	2	13	18	0(0)	2(11.1)	16(88.9)
Pre-immune*	2	7	8	0(0)	0(0)	8(100)
Anti-PH-30*§	4	31	51	3(5.9)	4(7.8)	44(86.3)
Anti-cPH-20*§	4	27	38	2(5.3)	0(0)	36(94.7)
Anti-mPH-20‡	4	39	79	73(92.4)	3(3.8)	3(3.8)
Anti-mPH-20*	4	42	83	82(98.8)	1(1.2)	0(0)

* Antibody pre-incubated with sperm and present in insemination drop.

‡ Antibody pre-incubated with sperm but not present in insemination drop.

§ Control antibodies. Anti-PH-30 is a rabbit antiserum that binds to another mouse sperm surface protein, PH-30. Anti-cPH-20 binds to mPH-20 (see Fig. 3 b) but does not inhibit mPH-20 hyaluronidase activity.

Discussion

The present study shows that the sperm protein PH-20 has a hyaluronidase activity and is present on the plasma membrane on the head of mouse and human sperm, being anchored in the membrane by a GPI anchor. Brief incubation of cumulus-enclosed mouse eggs with purified, recombinant PH-20 results in release of virtually all the cumulus cells surrounding the egg. The PH-20 hyaluronidase activity on the sperm plasma membrane is required for mouse sperm to penetrate the cumulus cell layer and reach the zona pellucida.

PH-20 Enzyme Activity and Membrane Location

The sequence of bee venom hyaluronidase was the first reported sequence of a eukaryotic hyaluronidase (9). The only other hyaluronidase sequence known is a hyaluronidase encoded by a bacteriophage that infects *Streptococcus pyogenes* (12). The bee venom and *S. pyogenes* sequences are unrelated (9). Since we found that PH-20 has hyaluronidase activity, the sequence homology between the bee venom enzyme and the amino terminal 307 amino acids of PH-20 should be a reflection of the conservation of enzymatic activity. There is 36% identity among amino acids in this first ~300 residues of the bee enzyme and PH-20. Because there is a large evolutionary distance between insects and mammals, the identical amino acid sequences between the bee hyaluronidase and the NH₂-terminal domain of PH-20 should represent some of the indispensable structural features of this type of hyaluronidase. PH-20's structural relationship to hyaluronidases from other mammalian tissues remains to be determined.

PH-20 is the only known membrane-anchored hyaluronidase. Our fluorescence microscopy showed that it is on the plasma membrane of both mouse and human sperm. The cDNA sequences of mPH-20 and hPH-20 predict a GPI-anchored protein (18 and Lathrop, W. F., E. P. Carmichael, D. G. Myles, and P. Primakoff. 1990. *J. Cell Biol.* 115:462a) and the PI-PLC release experiments indicated that both mPH-20 and hPH-20 do have a GPI anchor.

Function of PH-20 Hyaluronidase Activity in Sperm Penetration of the Cumulus Layer

The results that PH-20 has a hyaluronidase activity and is present on the sperm plasma membrane suggested that it might have a role in acrosome intact sperm penetrating the cumulus layer. Two kinds of experiment supported this idea.

First, we found that brief incubation of ovulated mouse eggs with purified PH-20 resulted in rapid release of the cumulus cells. Second, we developed an assay using R-123-labeled mouse sperm and confocal microscopy to evaluate the ability of sperm to pass through the cumulus layer and reach the zona. In this assay many sperm reach the zona within ten min after sperm addition to cumulus-enclosed eggs. This rapid penetration through the cumulus can be blocked by an anti-mPH-20 antibody that inhibits mPH-20 hyaluronidase activity (Table I, lines 5 and 6). In one type of experiment an acrosome intact sperm preparation was preincubated with the anti-mPH-20 antibody, and then pelleted and washed free of unbound antibody. When these preincubated sperm were added to an insemination drop containing eggs but no antibody, sperm penetration through the cumulus cell layer was blocked (Table I, line 5). This result indicates that pre-binding of the antibody to PH-20 on the plasma membrane of acrosome intact sperm is sufficient to block cumulus penetration. In this experiment the anti-mPH-20 antibody is absent in the insemination drop and thus antibody can not be inhibiting by binding to hyaluronidase released by sperm in the drop or by binding to sperm that acrosome react in the drop. The results from the cumulus penetration assays indicate that plasma membrane PH-20 is necessary for cumulus penetration. PH-20 (along with sperm motility) might also be sufficient for cumulus penetration because purified PH-20, in the absence of any other sperm component, can rapidly and completely remove the cumulus layer.

In the context of previous work on PH-20, it is a surprising finding that PH-20 has hyaluronidase activity and functions in an initial step of fertilization. In addition to mPH-20 and cPH-20, gpPH-20 (our unpublished results) has been found to have hyaluronidase activity. Previously gpPH-20 was shown to have a required function in the binding of acrosome reacted sperm to the zona (23, 27, 28), a later step in fertilization occurring after sperm penetration of the cumulus. We are currently investigating if PH-20 is a bifunctional protein with two distinct activities or if the hyaluronidase and sperm-zona adhesion activity are in some way related.

Significance

The presence of PH-20 hyaluronidase activity on the sperm plasma membrane of species from rodents to primates suggests that this enzyme activity has an essential, conserved function in vivo. One function is apparently in acrosome intact sperm penetration of the cumulus layer and appears to

be a fundamental feature of a mechanism that sperm use to reach the egg. This type of mechanism may be used by other cells that migrate through tissues. Such cells include neutrophils and lymphocytes migrating during immune responses and tumor cells migrating during metastasis. These cell types might express PH-20 or other plasma membrane-anchored hydrolases that could digest ECM components of the tissues into which the migrating cells penetrate.

In the study of the sequence of cellular events in fertilization, no satisfactory model has previously emerged that can integrate all the data. In an early model, sperm were thought to acrosome react outside the cumulus and then to be able to penetrate the cumulus because of the action of their released soluble hyaluronidase (20, 34, 41). A current paradigm, based on research findings of the past decade, states that a fertilizing, acrosome intact sperm passes through the cumulus layer, binds to the zona pellucida, and is then induced to acrosome react (1, 3, 7, 13, 21, 34, 39–41). This model has lacked an explanation for how acrosome intact sperm penetrate the cumulus (1, 41). Our data supply this missing element in the current model. Acrosome intact sperm can penetrate the cumulus layer using the hyaluronidase activity of plasma membrane PH-20, reach and bind to the zona, and then acrosome react on the zona and proceed to fertilize the egg.

We thank Gernot Walter, University of California (San Diego, CA) for supplying the KT3 mAb, Lida Kimmel for constructing the KT3-tagged cPH-20 clone, John Carson and Frank Morgan for invaluable assistance with the confocal microscope, and Lorri Goldsmith for editorial assistance.

Supported by National Institutes of Health grants 1U54-HD29123 (to P. Primakoff) and RO1-HD16580 (to D. G. Myles).

Note Added in Proof. After this paper was submitted the following paper was published: Gmachl, M., S. Sagan, S. Ketter, G. Kreil. 1993. "The human sperm protein PH-20 has hyaluronidase activity." *FEBS (Fed. Eur. Biochem. Soc.) Letts.* 336:545–548.

Received for publication 8 December 1993 and in revised form 7 March 1994.

References

- Bleil, J. D. 1991. Sperm Receptors of Mammalian Eggs. In *Elements of Mammalian Fertilization*. P. M. Wassarman, editor. CRC Press, Boca Raton, FL. 133–151.
- Bleil, J. D., and P. M. Wassarman. 1983. Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev. Biol.* 95:317–324.
- Cherr, G., H. Lambert, S. Meizel, and D. Katz. 1986. *In vitro* studies of the golden hamster sperm acrosome reaction: completion on the zona pellucida and induction by homologous soluble zonae pellucidae. *Dev. Biol.* 114:119–131.
- Cowan, A. E., P. Primakoff, and D. G. Myles. 1986. Sperm exocytosis increases the amount of PH-20 antigen on the surface of guinea pig sperm. *J. Cell Biol.* 103:1289–1297.
- Cummins, J. M., and R. Yanagimachi. 1986. Development of ability to penetrate the cumulus oophorus by hamster spermatozoa capacitated *in vitro*, in relation to the timing of the acrosome reaction. *Gamete Res.* 15:187–212.
- Dorfman, A. 1955. Mucopolysaccharidases. *Methods Enzymol.* 1:166–173.
- Drobnik, E. Z., and D. F. Katz. 1991. Videomicroscopy of mammalian fertilization. In *Elements of Mammalian Fertilization*. P. M. Wassarman, editor. CRC Press, Boca Raton, FL. 269–300.
- Florman, H. M., and B. T. Storey. 1982. Mouse gamete interactions: the zona pellucida is the site of the acrosome reaction leading to fertilization *in vitro*. *Dev. Biol.* 91:121–130.
- Gmachl, M., and G. Kreil. 1993. Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm. *Proc. Natl. Acad. Sci. USA.* 90:3569–3573.
- Harper, M. 1982. Sperm and egg transport. In *Germ Cells and Fertilization*. C. R. Austin and R. V. Short, editors. Cambridge University Press, Cambridge, U.K. 102–127.
- Hogan, B., F. Costantini, and E. Lacy. 1986. *Manipulating the Mouse Embryo*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 250 pp.
- Hynes, W. L., and J. J. Ferretti. 1989. Sequence analysis and expression in *Escherichia coli* of the hyaluronidase gene of *Streptococcus pyogenes* bacteriophage H4489A. *Infect. Immunol.* 57:533–539.
- Kopf, G. S., and G. L. Gerton. 1991. The mammalian sperm acrosome and the acrosome reaction. In *Elements of Mammalian Fertilization*. P. M. Wassarman, editor. CRC Press, Boca Raton, FL. 153–203.
- Kuppusamy, U. R., H. E. Khoo, and N. P. Das. 1990. Structure-activity studies of flavonoids as inhibitors of hyaluronidase. *Biochem. Pharmacol.* 40:397–401.
- Lathrop, W. F., E. P. Carmichael, D. G. Myles, and P. Primakoff. 1990. cDNA cloning reveals the molecular structure of a sperm surface protein, PH-20, involved in sperm-egg adhesion and the wide distribution of its gene among mammals. *J. Cell Biol.* 111:2939–2949.
- Deleted in proof.
- Lee, M. A., and B. T. Storey. 1985. Evidence for plasma membrane impermeability to small ions in acrosome-intact mouse spermatozoa bound to mouse zonae pellucidae, using an aminoacridine fluorescent pH probe: time course of the zona-induced acrosome reaction monitored by both chlortetracycline and pH probe fluorescence. *Biol. Reprod.* 33:235–246.
- Lin, Y., L. H. Kimmel, D. G. Myles, and P. Primakoff. 1993. Molecular cloning of the human and monkey sperm surface protein PH-20. *Proc. Natl. Acad. Sci. USA.* 90:10071–10075.
- MacArthur, H., and G. Walter. 1984. Monoclonal antibodies specific for the carboxy terminus of simian virus 40 large T antigen. *J. Virol.* 52:483–492.
- McRorie, R. A., and W. L. Williams. 1974. Biochemistry of Mammalian Fertilization. *Annu. Rev. Biochem.* 43:777–803.
- Miller, D. J., M. B. Macek, and B. D. Shur. 1992. Complementarity between sperm surface β 1,4-galactosyltransferase and egg coat ZP3 mediates sperm-egg binding. *Nature (Lond.)* 357:589–593.
- Myles, D. G., and P. Primakoff. 1984. Localized surface antigens of guinea pig sperm migrate to new regions prior to fertilization. *J. Cell Biol.* 99:1634–1641.
- Myles, D. G., H. Hyatt, and P. Primakoff. 1987. Binding of both acrosome-intact and acrosome-reacted guinea pig sperm to the zona pellucida during *in vitro* fertilization. *Dev. Biol.* 121:559–567.
- Phelps, B. M., P. Primakoff, D. E. Koppel, M. G. Low, and D. G. Myles. 1988. Restricted lateral diffusion of PH-20, a PI-anchored sperm membrane protein. *Science (Wash. DC)* 240:1780–1782.
- Phelps, B. M., D. E. Koppel, P. Primakoff, and D. G. Myles. 1990. Evidence that proteolysis of the surface is an initial step in the mechanism of formation of sperm cell surface domains. *J. Cell Biol.* 111:1839–1847.
- Primakoff, P., and D. G. Myles. 1983. A map of the guinea pig sperm surface constructed with monoclonal antibodies. *Dev. Biol.* 98:417–428.
- Primakoff, P., H. Hyatt, and D. G. Myles. 1985. A role for the migrating sperm surface antigen PH-20 in guinea pig sperm binding to the egg zona pellucida. *J. Cell Biol.* 101:2239–2244.
- Primakoff, P., W. Lathrop, L. Woolman, A. Cowan, and D. G. Myles. 1988. Fully effective contraception in male and female guinea pigs immunized with the sperm protein PH-20. *Nature (Lond.)* 335:543–546.
- Saling, P. M., and B. T. Storey. 1979. Chlortetracycline as a fluorescent probe for the mouse sperm acrosome reaction. *J. Cell Biol.* 83:544–555.
- Saling, P. M., J. Sowinski, and B. T. Storey. 1979. An ultrastructural study of epididymal mouse spermatozoa binding to zonae pellucidae *in vitro*: sequential relationship to the acrosome reaction. *J. Exp. Zool.* 209:229–238.
- Salustri, A., M. Yanagishita, and V. C. Hascall. 1990. Mouse oocytes regulate hyaluronic acid synthesis and mucification by FSH-stimulated cumulus cells. *Dev. Biol.* 138:26–32.
- Salustri, A., M. Yanagishita, C. Underhill, T. Laurent, and V. Hascall. 1992. Localization and synthesis of hyaluronic acid in the cumulus cells and mural granulosa cells of the preovulatory follicle. *Dev. Biol.* 151:541–551.
- Siiteri, J., P. Dandekar, and S. Meizel. 1988. Human sperm acrosome reaction-initiating activity associated with the human cumulus oophorus and mural granulosa cells. *J. Exp. Zool.* 246:71–80.
- Storey, B. Y., M. A. Lee, C. Muller, C. R. Ward, and D. G. Wirtshafter. 1984. Binding of mouse spermatozoa to the zonae pellucidae of mouse eggs in cumulus: evidence that the acrosomes remain substantially intact. *Biol. Reprod.* 31:1119–1128.
- Suarez, S., D. Katz, and S. Meizel. 1984. Changes in motility that accompany the acrosome reaction in hyperactivated hamster spermatozoa. *Gamete Res.* 10:253–265.
- Summers, M. D., and G. E. Smith. 1987. In *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*. 5–57.
- Talbot, P. 1985. Sperm penetration through oocyte investments in mammals. *Am. J. Anat.* 174:331–346.
- Talbot, P., G. DiCarantonio, P. Zao, J. Penkala, and L. T. Haimo. 1985. Motile cells lacking hyaluronidase can penetrate the hamster oocyte cumulus complex. *Dev. Biol.* 108:387–398.
- Ward, C., and G. Kopf. 1993. Molecular events mediating sperm activation. *Dev. Biol.* 158:9–34.
- Wassarman, P. M. 1993. Mammalian eggs, sperm and fertilisation: dissimilar cells with a common goal. *Sem. Dev. Biol.* 4:189–197.
- Yanagimachi, R. 1988. Mammalian fertilization. In *The Physiology of Reproduction*. E. Knobil and J. Neill, editors. Raven Press, Ltd., New York. 135–185.