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The PH-20 Protein in Human Spermatozoa

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ABSTRACT: PH-20 is a sperm plasma-membrane protein that has been shown to have hyaluronidase activity in several mammalian species including nonhuman primates. In this investigation, the PH-20 protein was characterized in noncapacitated human sperm and in capacitated human sperm. Two forms of PH-20 were observed in immunoblots of sodium dodecylsulfate polyacrylamide-gel electrophoresis (SDS PAGE) using a polyclonal antibody to recombinant PH-20: a major band of 64 kDa appeared in noncapacitated and capacitated sperm extracts and a 53-kDa band that appeared only in the acrosome-reaction supernatant of acrosome-reacted sperm. Using hyaluronic acid substrate gel analysis, we demonstrated that noncapacitated sperm extracts, capacitated sperm extracts, and the acrosome-reaction supernatant had hyaluronidase activity at neutral pH (pH 7) and acid pH (pH 4). The 64-kDa form in all samples had hyaluronidase activity at both neutral and acid pH, but the 53-kDa form was only active at acid pH. Total hyaluronidase activity, as measured by a microplate assay, was higher at pH 7 than at pH 4. Very low hyaluronidase activity was detected in the acrosome-reaction supernatant. Transmission electron microscopy and immunogold labeling showed that PH-20 of acrosome-intact human sperm was located on the plasma membrane over the entire head but not on the sperm midpiece and tail. After the acrosome reaction, PH-20 was also located on the inner acrosomal membrane. The biochemical characteristics and the ultrastructural localization of PH-20 in human sperm suggest that this protein is the human sperm hyaluronidase and, therefore, has an important function during fertilization.

Key words: Sperm, hyaluronidase, fertilization, acrosome reaction, capacitation.

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It has been known for many years that mammalian sperm contains hyaluronidase (Mann, 1964). This acrosomal enzyme has been detected in numerous species (Stambaugh and Buckley, 1970; Brown, 1975; Morton, 1976), and multiple forms of the enzyme have been described (Harrison, 1988). Hyaluronidase has been shown to be released during the acrosome reaction (Shams-Borhan and Harrison, 1981), but only recently has it been suggested that a sperm plasma membrane-associated hyaluronidase could be involved in penetration of the cumulus oophorus (Cummins and Yanagimachi, 1986; Yudin et al, 1988; Lin et al, 1994; Thaler and Cardullo, 1995). Evidence has been presented that indicates that PH-20, a glycosyl phosphatidylinositol (GPI)-anchored sperm membrane protein, is responsible for sperm-surface hyaluronidase activity (Gmachl et al, 1993; Lin et al, 1994; Cherr et al, 1996). This possibility became apparent when the PH-20 cDNA was cloned and it was found to have significant sequence homology with bee venom hyaluronidase (Lathrop et al, 1990; Gmachl and Kreil, 1993). The PH-20 gene has been shown to be present in many species including mouse, rat, hamster, bovine, macaque, and human (Lathrop et al, 1990). Recombinant PH-20 has been produced, and antibodies against the purified protein have been generated (Lin et al, 1993, 1994). These antibodies have been used to demonstrate the presence of a protein that is homologous to PH-20 on the plasma membrane of acrosome-intact mouse and human sperm (Lin et al, 1994), as well as on the macaque sperm (Overstreet et al, 1995). The PH-20 of macaque sperm has been localized to the plasma membrane overlying the anterior and posterior sperm head as well as to the inner acrosomal membrane and the soluble contents that are released during the acrosome reaction (Overstreet et al, 1995; Cherr et al, 1996). All of the hyaluronidase activity in cynomolgus macaque sperm can be attributed to PH-20, but the PH-20 enzyme appears to have several forms that are biochemically and functionally distinct (Cherr et al, 1996). These forms include enzymes that are primarily active at neutral pH (64 kDa form) and at acid pH (53 kDa form). It appears that the soluble form of macaque PH-20 is active at acid pH and may result from enzymatic processing of the 64-kDa form to the 53-kDa form, which is released from sperm after the acrosome reaction (Cherr et al, 1996).

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PH-20 is a possible target for immunocontraception in humans, and vaccines directed against this antigen are being tested for contraceptive efficacy in macaques. It is important to verify that the PH-20 proteins in human sperm and macaque sperm have similar biochemical characteristics because species differences in PH-20 may exist (Harrison et al, 1988). In this communication, we report our observations of PH-20 in noncapacitated and capacitated human sperm with respect to its molecular weight, hyaluronidase activity, and cellular localization.

Materials and Methods

Materials

All chemicals used were reagent grade. Bovine serum albumin (BSA), polyvinyl alcohol (PVA), Percoll, and cetyl pyridium chloride (CPC) were purchased from Sigma (St. Louis, Missouri). The following electrophoresis-grade chemicals were purchased from Bio-Rad (Hercules, California): ammonium persulfate, sodium dodecylsulfate (SDS), biotinylated molecular weight standards, and goat antirabbit immunoglobulin G (IgG). A sepraAcryl-30 solution containing 30:0.8 acrylamide : Bis was purchased from Integrated Separation Systems (Natick, Maryland); hyaluronic acid was purchased from Calbiochem (La Jolla, California), and Wydase® from Wyeth-Ayerst, Inc. (Philadelphia, Pennsylvania).

Sperm Preparation and Treatments

Sperm Washing and Capacitation—Human semen was obtained by masturbation from healthy donors. Liquified semen samples were centrifuged at 300 × *g* for 20 minutes on a two-step Percoll gradient (80%/40%). This procedure yields a population of >95% motile sperm. The medium used for the Percoll gradient and for the subsequent washing step was a modified Biggers Whitten and Whittingham (BWW) medium (Overstreet et al, 1980) containing 2 mM glucose, 0.25 mM pyruvate, 19 mM lactate, 3 mg/ml BSA, 750 units/ml streptomycin sulfate, and 1,670 units/ml penicillin G. The sperm recovered after centrifugation were defined as noncapacitated and were prepared for morphological and biochemical analysis as described below. To obtain capacitated sperm, the pellets from the Percoll gradient were resuspended and diluted to a concentration of 6×10^6 sperm/ml in the medium described above except with 35 mg/ml BSA. Resuspended sperm were capacitated by incubating 500 μl aliquots in 15-ml polystyrene conical centrifuge tubes (Fisher Scientific, Fair Lawn, New Jersey) at 37°C for 6 hours in a humid atmosphere of 5% CO_2/95% air (pH 7.4–7.6).

Induction of Acrosome Reactions—After 6 hours capacitation, sperm were pooled in 2 ml and centrifuged at 300 × *g* for 10 minutes on 40% Percoll to remove the BSA. The washed sperm were resuspended in the same buffer medium with metabolites and 0.1% PVA in place of BSA. Half of the sample was acrosome reacted by addition of the calcium ionophore, A23187 [15 μM in dimethylsulfoxide (DMSO)], for 10 minutes. The sperm were resuspended at a concentration of 200×10^6 sperm/ml for SDS polyacrylamide-gel electrophoresis (SDS PAGE). A small aliquot (5 μl) was used for determination of the percentage of motile sperm and for subjective estimates of the quality of sperm motility (Thomas and Meizel, 1988). The percentage of acrosome reactions was determined by the method of Cross et al (1986) as modified by Overstreet et al (1995). Sperm were fixed in 4% formaldehyde and in ethanol, and the percentage of acrosome-reacted sperm was determined using fluorescein isothiocyanate-conjugated Pisum sativum agglutinin (Vector Laboratories, Burlingame, California) (100 μg/ml). Two hundred sperm were counted in each sample to determine the percentage of sperm that were acrosome-reacted according to the criteria of Cross et al (1986).

Sperm Preparation for Substrate Gel Electrophoresis and Immunoblotting—Noncapacitated or capacitated sperm were washed by centrifugation in BWW medium containing 0.1% PVA without BSA and resuspended at a concentration of 200×10^6 sperm/ml. Noncapacitated sperm and one-half of the capacitated sample (whole washed sperm) were aliquoted and combined with protease inhibitors (0.05 M Tris, pH 7.4, 0.02 M EDTA, 1 mM *p*-hydroxymercurobenzoate, 5 mM *N*-ethylmaleimide, and 1 mM paraamino-benzamidine). The other half of the capacitated sperm sample was acrosome reacted with 15 μM calcium ionophore as described above. Protease inhibitors were added, as above, to the acrosome-reaction mixture. Both acrosome-intact samples and acrosome-reacted samples were centrifuged at 10,000 × *g* for 10 minutes. The 10,000 × *g* acrosome-intact sperm pellet was resuspended in buffer containing the protease inhibitors. The supernatant of the acrosome-reacted sample was recovered and re-centrifuged at 100,000 × *g* for 60 minutes to obtain a soluble fraction (referred to as the acrosome-reaction supernatant). Sperm pellets were solubilized in 2% Triton X-100, then vortexed vigorously for 5 minutes. The samples were then run on SDS PAGE for hyaluronic acid-gel substrate analyses and western blot analyses. For each experiment described, the assay was performed three times. For the western blots, three or four samples were pooled, then divided to provide suspensions of noncapacitated, capacitated, and acrosome-reacted sperm. These experiments also were repeated three times.

SDS-Gel Electrophoresis and Western Blot Analysis—Sperm samples were solubilized with 4× nonreducing SDS sample buffer consisting of Tris, SDS, glycerol, and bromophenol blue. Half of each sample was heated to 95°C for 5 minutes prior to the western blot analysis while the other half was used without heating for the hyaluronic acid-substrate-gel analysis. A small sperm aliquot was dissolved in SDS sample buffer with no β-mercaptoethanol or bromophenyl blue for spectrophotometric quantitation of protein concentration using the bicinchoninic acid method (BCA kit, Pierce, Rockford, Illinois).

Electrophoresis by SDS PAGE under non-reducing conditions was carried out with a mini-gel electrophoresis unit (Bio-Rad Corp., Hercules, California) on 7.5% acrylamide-gel slabs. Broad-range biotinylated protein standards with apparent molecular weights of 6,500–200,000 were electrophoresed in parallel with the sperm extracts. Recombinant cynomolgus macaque PH-20 was used as a positive control. The separated proteins were electrophoretically transferred to nitrocellulose membranes (Towbin et al, 1979) and incubated for 5 hours in a blocking solution, 3% gel Tris-buffered saline–Tween 20 (TBST) solution

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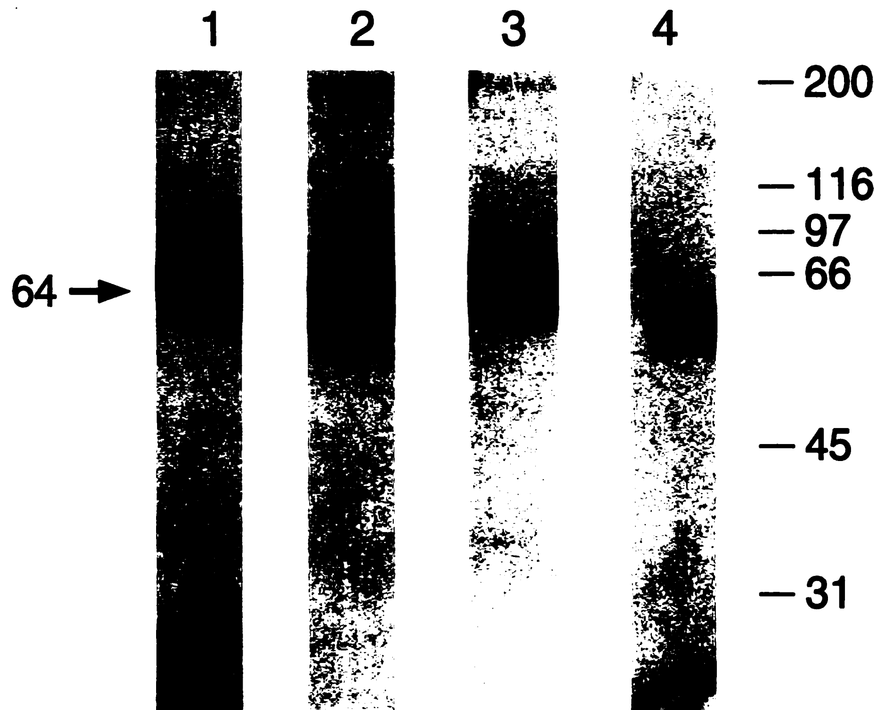

FIG. 1. Immunoblots of SDS PAGE of human sperm boiled in nonreducing SDS sample buffer and probed with antibody raised in rabbits to recombinant macaque PH-20. (Lane 1) Solubilized capacitated acrosome-reacted sperm. (Lane 2) Solubilized capacitated acrosome-intact sperm. Note 63–66 kDa immunoreactive band. (Lane 3) Solubilized washed noncapacitated sperm. (Lane 4) Recombinant macaque PH-20. Molecular-weight standards were myosin, 200 kDa; β galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa.

consisting of 10 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.4, then probed with antisera raised in rabbits (R-10) against purified recombinant cynomolgus macaque PH-20 (Lin et al, 1994) or control rabbit serum, for 20 hours at room temperature. After four subsequent washes (15 minutes each) in 50 ml of TBST solution without antibody, the membranes were incubated for 2 hours with the secondary antibody, an antirabbit IgG conjugated to horseradish peroxidase (used at 1:3,000 in the 1% gel-TBST solution). The membrane blots were visualized with horseradish peroxidase (HRP) color-development reagents (Bio-Rad, Hercules, California) or with fluorescent electrogenerated chemiluminescence (ECL) kit (Amersham, Arlington Heights, Illinois). Biotinylated molecular-weight standards were observed using an antibody to biotin conjugated to HRP.

Hyaluronic Acid-Substrate-Gel Electrophoresis—Substrate-gel electrophoresis of sperm was conducted according to Guntenhoner et al (1992). Briefly, 7.5% SDS polyacrylamide gels were prepared containing 170 μg/ml hyaluronic acid in the separating gel prior to polymerization. The stacking gel (4.5%) did not contain hyaluronic acid. Following electrophoresis, gels were incubated at room temperature for 2 hours in TBS containing 3% Triton X-100 in order to remove SDS. Gels were then incubated in 100 mM sodium acetate buffer at pH 4.0 or in Tris buffer at pH 7.0 for 18–20 hours at 37°C. To visualize regions of hyaluronic acid digestion in the gels, they were stained with 0.5% Alcian blue in 3% acetic acid for 2 hours, destained in 7% acetic acid, and counterstained with Coomassie blue R-250.

Microplate Assay for Hyaluronidase Activity—Enzyme activity of noncapacitated and capacitated acrosome-intact or acrosome-reacted sperm and the acrosome-reaction supernatant from the acrosome-reacted sperm was determined using a microplate assay according to a method described by Tung et al (1994). For this assay, hyaluronic acid is suspended in agarose in a microtiter well, the plate is incubated with the test solution, and the undigested hyaluronic acid is precipitated with cetylpyridinium chloride. The precipitate blocks the light transmittance; therefore, an increase in the visible light transmitted correlates with the amount of digested hyaluronic acid. Briefly, hyaluronic acid was dissolved in water at a concentration of 8 mg/ml and maintained at 55°C before use. The hyaluronic acid solution was mixed with the agarose to give a final concentration of 0.8 mg/ml of hyaluronic acid and 0.8% (w/v) of agarose. The 96-well microtiter plates (Nunc, Inc., Naperville, Illinois) were prewashed 3× with the appropriate buffers at pH 4 or pH 7. Warm hyaluronic acid–agarose mixture (100 μl) was dispensed into each well of the microplate. Once the gel had set, each well was filled with 100 μl of the hyaluronidase (standards or unknown samples). Hyaluronidase activity was determined relative to a standard curve established using serial dilutions of a commercial bovine testicular hyaluronidase preparation (Wydase®) at pH 7.0 in 0.05 M Tris buffer. Standards and unknown samples were determined in triplicate for each assay. For detection of hyaluronidase activity at acid and neutral pH, the Triton X-100 extracts of sperm samples were thawed at 4°C and diluted 1:20 in 0.1 M sodium formate buffer (pH 4.0) or 0.05 M Tris buffer (pH 7.0), respectively. Following incubation for 15 hours at 37°C, the microplate wells were washed 3× with the appropriate buffer. Each well was then filled with 100 μl of 10% (w/v) aqueous CPC. The

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absorbance at 595 nm was measured using an automated microplate reader (Dynatech, Inc., Alexandria, Virginia). Triplicate control wells containing no hyaluronidase were incubated with or without CPC to determine maximum and minimum optical densities, respectively. Computer software (DeltaSoft II, Biometallics, Princeton, New Jersey) was used to calculate unknown sample enzyme activity in National Formulary Units (NFU) relative to the Wydase standard curve at pH 7.0. Hyaluronidase activity was expressed as relative NFUs after a four-parameter fit was determined for each assay (average $r^2 = 0.95$). Data were analyzed using the one-tailed Dunnett t-test for multiple comparisons. Values of $P \leq 0.05$ were considered significantly different.

Ultrastructural Localization of PH-20

For immunolabelling, sperm suspensions were fixed for 20 minutes in phosphate-buffered saline (PBS) buffered 4% paraformaldehyde at room temperature and then were centrifuged at $1{,}000 \times g$ for 3 minutes. Pelleted sperm were washed twice by centrifugation and resuspended in 1 ml of Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, New York) containing 1% BSA (blocking buffer). Sperm suspensions were incubated with rabbit anti-PH-20 antiserum or control rabbit serum (1:25) for 2 hours. Sperm suspensions were again washed twice in blocking buffer. Secondary gold labelling was accomplished with gold-conjugated antirabbit IgG (E.Y. Lab., San Mateo, California) diluted 1:20 with blocking buffer. After incubation for 2 hours, the sperm samples were pelleted and resuspended in 2.5% glutaraldehyde buffered in 0.1 M cacodylate (pH 7.4) according to a method previously described (Overstreet et al, 1995). After fixation, the samples were washed twice for 30 minutes. Washed samples were dehydrated through a graded ethanol series. The sperm suspensions were further dehydrated in 100% acetone before embedding in Spurr's epoxy (Ted Pella, Inc., Redding, California). Spurr's embedded sperm were centrifuged into an epon–araldite epoxy. Sections were cut on a diamond knife and were stained with uranyl acetate and lead citrate before being viewed on a Phillips 410 transmission electron microscope.

Results

We evaluated the characteristics of PH-20 protein in four different human sperm samples: washed, uncapacitated sperm, 6-hour capacitated, acrosome-intact sperm, acrosome-reacted sperm, and the soluble material released at the time of the acrosome reaction. The samples were extracted in nonreducing SDS solubilization buffer and immediately boiled for 5 minutes prior to SDS PAGE. In preparations of noncapacitated sperm, the anti-PH-20 polyclonal antibody recognized a diffuse band with apparent molecular weight of 64 kDa (Fig. 1). In addition, extracts from capacitated, acrosome-intact sperm, and acrosome-reacted sperm (45–55% acrosome reacted) also showed a diffuse band of 64 kDa when probed with this antibody (Fig. 1). On the other hand, the acrosome-reaction supernatant included an additional smaller band at 53 kDa (Fig. 2). This band was only visible on the western blot when detected with the ECL kit but not with the conventional HRP color-development reagents. Western blots with control rabbit serum showed no evidence of these bands (data not shown).

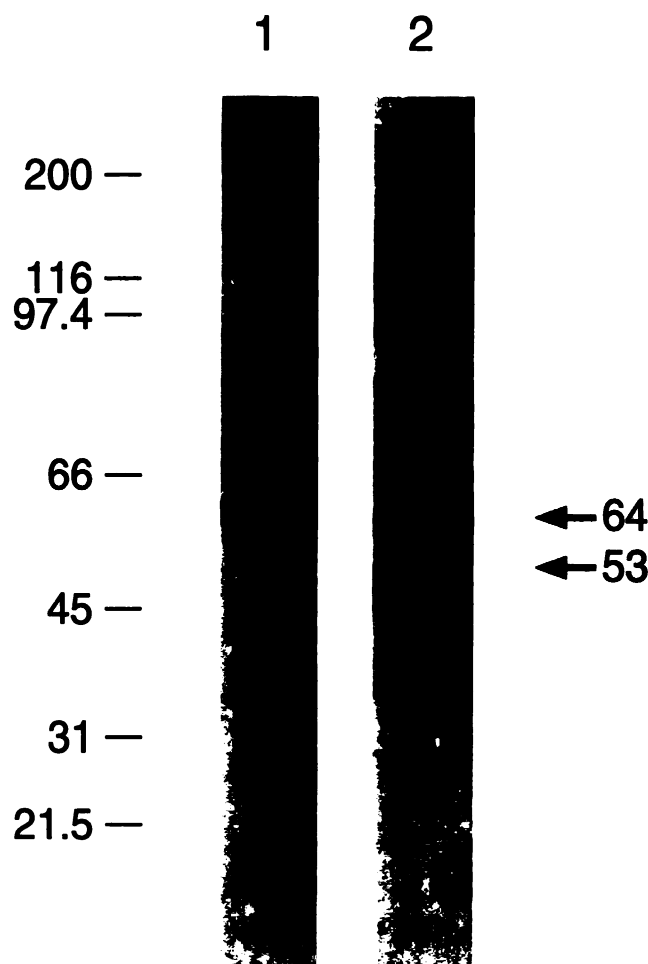

FIG. 2. Immunoblots of SDS PAGE of human sperm boiled in nonreducing SDS sample buffer and probed with antibody raised in rabbits to recombinant macaque PH-20. (Lane 1) Solubilized capacitated acrosome-reacted sperm. (Lane 2) Acrosome-reaction supernatant from acrosome-reacted sperm. Note 53 kDa immunoreactive band.

Hyaluronidase activity associated with PH-20 was measured using hyaluronic acid-substrate-gel analysis of noncapacitated sperm, capacitated, acrosome-intact sperm, and the acrosome-reaction supernatant from the acrosome-reacted sperm. The hyaluronidase activity of all three sperm samples was associated with the 64-kDa form of PH-20 (Fig. 3) at physiological pH and acid pH. The 64-kDa form of PH-20 appeared with a higher intensity at pH 7 than at pH 4, even though the same amount of protein was loaded into each lane. This finding was observed consistently in all three experiments. The 64-kDa form of PH-20 in the acrosome-reaction supernatant also

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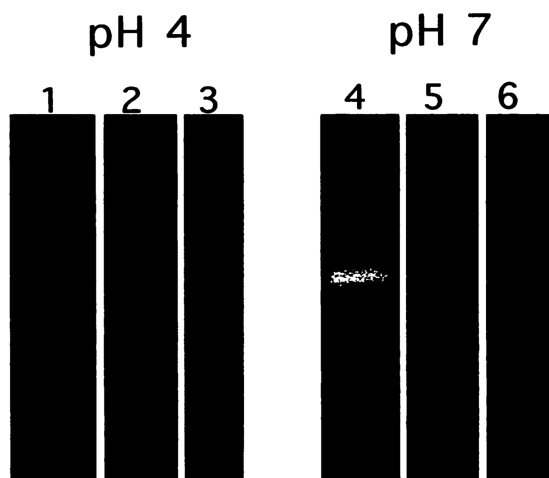

FIG. 3. Hyaluronic acid substrate SDS PAGE (7.5% gels) of human-sperm samples at pH 4 or pH 7. (Lane 1) Solubilized washed noncapacitated sperm extract, pH 4. (Lane 2) Solubilized capacitated-sperm extract, pH 4. (Lane 3) Acrosome-reaction supernatant from acrosome-reacted sperm, pH 4. (Lane 4) Solubilized washed noncapacitated sperm extract, pH 7. (Lane 5) Solubilized capacitated sperm extract, pH 7. (Lane 6) Acrosome-reaction supernatant from acrosome-reacted sperm, pH 7.

had hyaluronidase activity at both acid and neutral pH, but the 53-kDa form was active only at acid pH (Fig. 3). Acrosome-intact and acrosome-reacted sperm gave the same results in the hyaluronic acid-substrate-gel analyses (data not shown).

Total hyaluronidase activity (measured by the microplate assay) was significantly higher ($P < 0.05$) at pH 7 than at pH 4 in all sperm extracts (Fig. 4). Very low hyaluronidase activity was detected in the acrosome-reaction supernatant, regardless of the pH. Hyaluronidase activity of the acrosome-reaction supernatant only represented a small percentage of total activity when compared to noncapacitated sperm (12.8% at pH 7 and 10.2% at pH 4, see Fig. 4). The microplate assay did not detect any significant difference in hyaluronidase activity of the acrosome-reaction supernatant at pH 4 and pH 7.

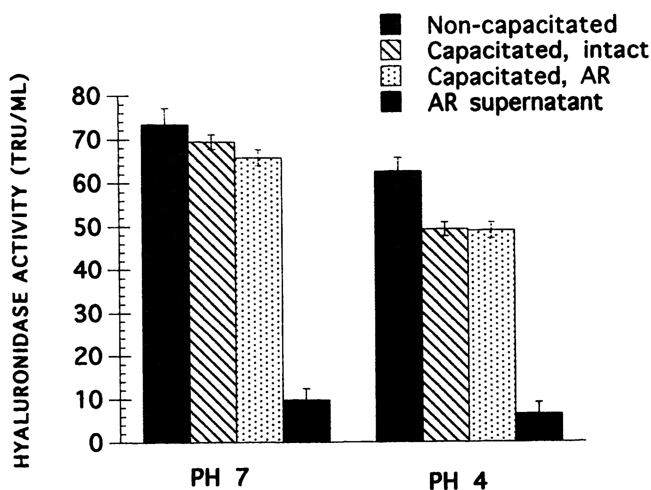

FIG. 4. Microplate hyaluronidase assay for Triton X-100 extracts of human sperm (noncapacitated); after 6 hours capacitation, acrosome-intact sperm (capacitated intact), acrosome-reacted sperm (capacitated, AR), and the acrosome-reaction supernatant (AR supernatant) at pH 7 and pH 4. Data are expressed as mean value ± SEM (n = 3).

Capacitated sperm, probed for PH-20 with polyclonal antibodies to recombinant macaque PH-20 and a secondary gold label, revealed a continuous uniform distribution of gold particles over the entire sperm head (Fig. 5). A similar labeling pattern was observed on noncapacitated sperm (data not shown). Little or no label was associated with the midpiece or flagellum nor was there any labeling of sperm exposed to control rabbit sera (data not shown). There was no evidence for regionalization of PH-20 on the sperm-head surface at the anterior head, equatorial segment, and posterior head (Fig. 5).

Soon after the acrosome reaction, the gold particles were found associated with the posterior head and equatorial segment (Fig. 6), as seen in the acrosome-intact sperm, but gold particles also were associated with the inner acrosomal membrane and the acrosomal vesicles derived from fusion of the plasma membrane and the outer acrosomal membrane (Fig. 6).

Discussion

In the present study, two forms of PH-20 were observed in human-sperm immunoblots of nonreducing SDS PAGE: a major band of 64 kDa that appeared in extracts of capacitated sperm and noncapacitated sperm and a minor band of 53 kDa that appeared only in the acrosome-reaction supernatant of acrosome-reacted sperm. These results are consistent with the previous observations of macaque sperm PH-20 (Cherr et al, 1996) and guinea pig sperm PH-20 (Primakoff et al, 1988). The 64-kDa form of PH-20 migrates as a diffuse band. This behavior on SDS PAGE could be due to the glycoprotein structure of PH-20 as discussed by Cherr et al (1996), who speculated that the appearance of the 53-kDa form of macaque PH-20 might be due to glycosidase cleavage of carbohydrate residues of the 64-kDa form. It is also possible that other proteases and even phospholipases are responsible for cleavage and/or release of PH-20 from the membrane, as occurs for other GPI-anchored proteins. The 53-kDa form of PH-20 in human sperm appeared only in the acrosome-reaction supernatant recovered after the induction of acrosome reactions with the calcium ionophore.

In contrast to macaque sperm PH-20, human sperm PH-20 appeared to be relatively resistant to breakdown by endogenous sperm proteases. In macaque sperm, the 53-kDa form was present in all sperm extracts that were not boiled immediately after addition of solubilization

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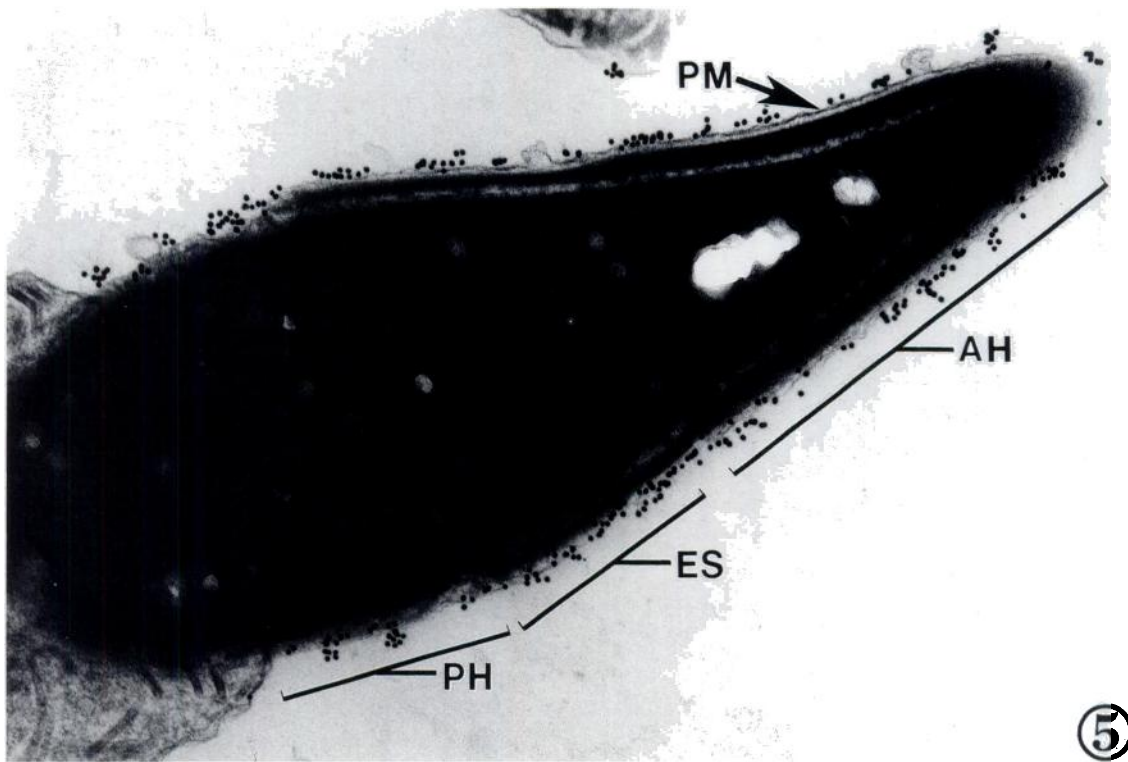

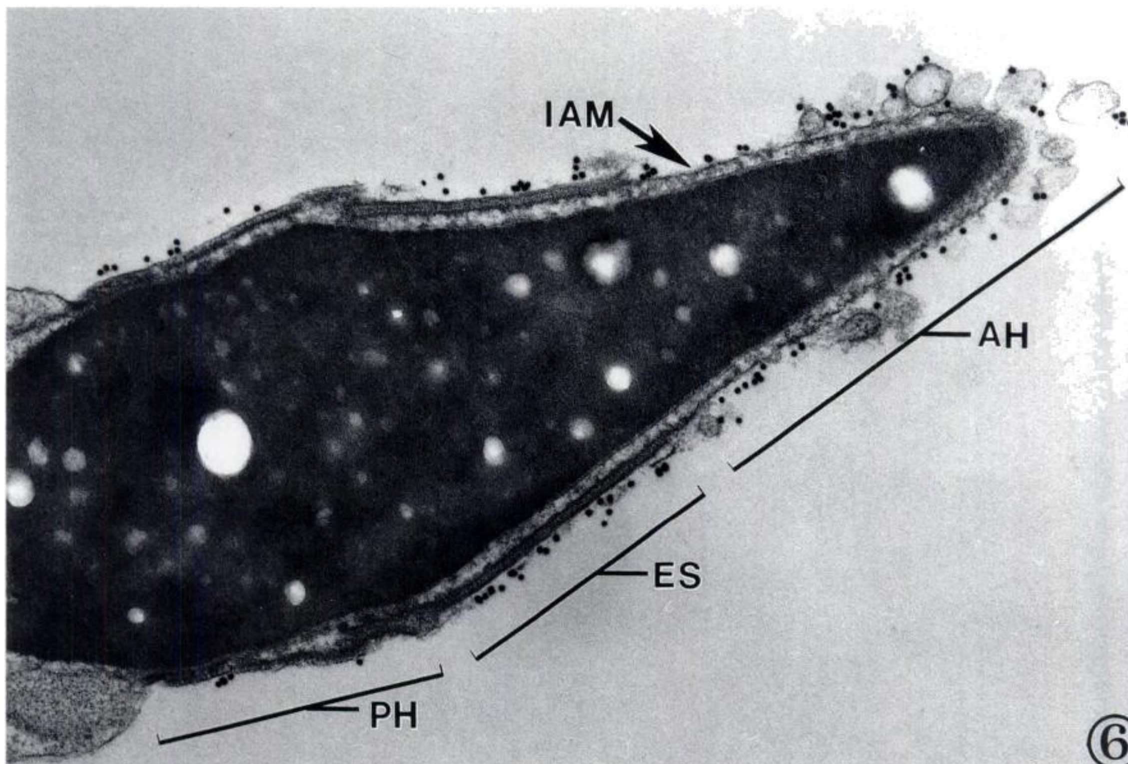

FIG. 5. Fine structural immunolocalization of PH-20 on acrosome-intact human sperm. Gold particles uniformly labeled the plasma membrane (PM) of the sperm head, including anterior head (AH), equatorial segment (ES), and posterior region of the head (PH).

FIG. 6. Fine structural immunolocalization of PH-20 on acrosome-reacted human sperm recovered 10 minutes after treatment with A23187. After acrosome reaction, gold particles are also associated with the inner acrosomal membrane (IAM).

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buffer, as well as in the acrosome-reaction supernatant (Cherr et al, 1996). Although the data reported in the present study were obtained with a protocol that included immediate boiling, we observed only the 64-kDa form in whole-sperm extracts, even when boiling was omitted prior to solubilization (data not shown). This greater resistance of human PH-20 to proteolysis may explain why we never observed the 53-kDa form in whole-sperm extracts after 6 hours capacitation; whereas, Cherr et al (1996) reported that the lower molecular-weight form could be demonstrated in acrosome-reacted macaque sperm as well as in the acrosome-reaction supernatant. It is possible that the 53-kDa form observed by Cherr et al (1996) in whole acrosome-reacted sperm was due to proteolytic degradation that did not occur in human-sperm preparations. Alternatively, the acrosomal shroud (membrane vesicles and matrix) that is present in acrosome-reacted macaque sperm (unpublished observations), but is not present in acrosome-reacted human sperm (Yudin et al, 1988), could have included the soluble 53-kDa form of PH-20 trapped within the matrix material.

It was shown previously that recombinant human PH-20 exhibits hyaluronidase activity (Gmachl et al, 1993). In the present study, hyaluronidase activity of both noncapacitated human sperm and capacitated, acrosome-intact human sperm was associated with the 64-kDa form of PH-20 that was active at both acid and neutral pH but had significantly more activity at neutral pH. Acrosome-intact macaque sperm also have the majority of their hyaluronidase activity at physiological pH, and this activity is associated with the 64-kDa form of PH-20 (Cherr et al, 1996).

The lower molecular-weight form of PH-20 (53 kDa) that was observed only in the acrosome-reaction supernatant did not show any hyaluronidase activity on the substrate gel at neutral pH. This is consistent with the activity of this form of PH-20 in macaque sperm (Cherr et al, 1996). However, in contrast to macaque sperm, the 64-kDa form of human PH-20 was active in the acrosome-reaction supernatant and had hyaluronidase activity at both neutral and acid pH. The reason for this apparent species difference is not entirely clear. The 64-kDa form of macaque sperm PH-20 is also present in the acrosome-reaction supernatant but has no detectable hyaluronidase activity on substrate gel (Cherr et al, 1996). The activity of the 64-kDa form of human PH-20 in the acrosome-reaction supernatant may be related to the apparent resistance of the human protein to endogenous proteolysis at the time of the acrosome reaction, a property that could also affect enzyme activity.

In control experiments, the supernatant from capacitated, acrosome-intact human sperm was evaluated with the same methods used for acrosome-reacted sperm, and these preparations had no evidence of the 64-kDa form PH-20 (data not shown). This finding indicates that the 64-kDa protein that was present in the acrosome-reaction supernatant was not released from the plasma membrane of capacitated acrosome-intact sperm.

The preservation of enzyme activity of the 64-kDa form of human PH-20 in the acrosome-reaction supernatant may also explain why we saw no quantitative differences in hyaluronidase activity (in the microplate assay) of the acrosome-reaction supernatant at pH 4 and pH 7. The majority of macaque PH-20 in the acrosome-reaction supernatant is acid active, but the 64-kDa form appears to contribute little to this activity (Cherr et al, 1996). The persistent activity of the 64-kDa form of human PH-20 in the acrosome-reaction supernatant is probably responsible for the majority of hyaluronidase activity detected at pH 7.

The hyaluronidase activity of macaque sperm PH-20 appears quantitatively greater in the acrosome-reaction supernatant than in the whole-sperm extracts (Cherr et al, 1996), while our results with human sperm indicate that hyaluronidase activity in the whole sperm extracts is 5–10× greater than in the acrosome-reaction supernatant. Some of this discrepancy may be due to the different methods used in the two studies for quantitative assay of hyaluronidase activity. An additional difference between the two species is the level of acrosome reactions that can be induced with calcium ionophore in capacitated sperm. In the present study, the percentage of acrosome reactions ranged from 45% to 55% in preparations used to recover the soluble acrosomal contents. In macaques, approximately 80% of sperm were acrosome reacted following ionophore treatment (Cherr et al, 1996). However, it is possible that the relative amount of soluble PH-20 released by human sperm, as a consequence of the acrosome reaction, is less than the amount released by macaque sperm.

PH-20 has been shown to be present on the human sperm with immunofluorescence (Lin et al, 1994). We studied the localization of PH-20 in human sperm before and after the acrosome reaction using transmission electron microscopy and immunogold labeling. Our ultrastructural observations are consistent with previous studies of macaque sperm (Overstreet et al, 1995) and demonstrate that PH-20 of acrosome-intact human sperm is located on the plasma membrane over the entire head and, also, on the inner acrosomal membrane.

PH-20 could function enzymatically through its hyaluronidase activity to facilitate passage of sperm through the cumulus. Meyers et al (1996) showed that antibodies to PH-20 or hyaluronidase inhibitors could inhibit macaque-sperm penetration into the cumulus. Lin et al (1994) showed a similar inhibition by anti-PH-20 antibodies of cumulus penetration by acrosome-intact mouse sperm. The presence of PH-20 on the inner acrosomal

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membrane suggests that this protein could have a function in secondary sperm zona binding or during sperm penetration through the zona pellucida. In this role, the protein may function nonenzymatically as a zona-binding molecule.

The multiple functions of PH-20 and its location on the sperm surface make it an attractive target for contraceptive vaccines (Meyers et al, 1996). The results of this investigation suggest that the PH-20 protein may have similar functions in human sperm and macaque sperm. Similar forms of PH-20 were observed in immunoblots of human and macaque sperm (64 and 53 kDa), and the protein had a similar ultrastructural localization in both species. These findings suggest that the macaque sperm is a good model for mechanistic studies of the effects of contraceptive vaccines that target the PH-20 protein. If a vaccine against PH-20 can be shown to be effective in reducing the fertility of macaques, then it is likely that similar contraceptive effects will be obtained with an anti-PH-20 vaccine in humans.

References

Brown CR. Distribution of hyaluronidase in the ram spermatozoon. *J Reprod Fertil* 1975;45:537–539.

Cherr GN, Meyers SA, Yudin AI, VandeVoort CA, Myles DG, Primakoff P, Overstreet JW. The PH-20 protein in cynomolgus macaque spermatozoa: identification of two different forms exhibiting hyaluronidase activity. *Dev Biol* 1996;175:142–153.

Cowan AE, Primakoff P, Myles DG. Sperm exocytosis increases the amount of PH-20 antigen on the surface of guinea pig sperm. *J Cell Biol* 1986;103:1289–1297.

Cross NL, Morales P, Overstreet JW, Hanson FW. Two simple methods for detecting acrosome-reacted human sperm. *Gam Res* 1986;15:213–226.

Cummins JH, Yanagimachi R. Development of ability to penetrate the cumulus oophorus by hamster spermatozoa capacitated *in vitro* in relation to the timing of the acrosome reaction. *Gam Res* 1986;15:187–212.

Gmachl M, Kreil G. Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm. *Proc Natl Acad Sci USA* 1993;90:3569–3573.

Gmachl M, Sagan S, Ketter S, Kreil G. The human sperm protein PH-20 has hyaluronidase activity. *FEBS Lett* 1993;336:545–548.

Guntenhoner MW, Pogrel MA, Stern R. A substrate gel assay for hyaluronidase activity. *Matrix* 1992;12:388–396.

Harrison RAP. Preliminary characterization of multiple forms of ram sperm hyaluronidase. *Biochem J* 1988;252:875–882.

Lathrop WF, Carmichael EP, Myles DG, Primakoff P. 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* 1990;111:2939–2949.

Lin Y, Kimmel LH, Myles DG, Primakoff P. Molecular cloning of the human and monkey sperm surface protein PH-20. *Proc Natl Acad Sci USA* 1993;90:10071–10075.

Lin Y, Maham K, Lathrop WF, Myles DG, Primakoff P. A hyaluronidase activity of the sperm membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. *J Cell Biol* 1994;125:1157–1163.

Mann T. *The Biochemistry of Semen and of the Male Reproductive Tract.* London: Methuen; 1964.

Meyers SA, Yudin AI, Cherr GN, VandeVoort CA, Myles DG, Primakoff P, Overstreet JW. Hyaluronidase activity of macaque sperm assessed by an *in vitro* cumulus penetration assay. *Mol Reprod Dev* 1996; in press.

Morton DB. Lysosomal enzymes in mammalian spermatozoa. In: Dingle JT, Dean RT, eds. *Lysosomes in Biology and Pathology.* Vol 5. Amsterdam: North Holland; 1976:203–255.

Overstreet JW, Lin Y, Yudin AI, Meyers SA, Primakoff P, Myles DG, Katz DF, Vandevoort CA. Location of the PH-20 protein on acrosome-intact and acrosome-reacted spermatozoa of cynomolgus macaques. *Biol Reprod* 1995;52:105–114.

Overstreet JW, Yanagimachi R, Katz DF, Hayashi K, Hanson FW. Penetration of human spermatozoa into the human zona pellucida and zona-free hamster egg: a study of fertile donors and infertile patients. *Fertil Steril* 1980;33:534–542.

Primakoff P, Cowan A, Hyatt H, Tredick-Kline J, Myles DG. Purification of the guinea pig sperm PH-20 antigen and detection of a site-specific endoproteolytic activity in sperm preparations that cleave PH-20 into two disulfide-linked fragments. *Biol Reprod* 1988;38:921–934.

Shams-Borhan G, Harrison RAP. Production, characterization and use of ionophore-induced calcium dependent acrosome reaction in ram spermatozoa. *Gam Res* 1981;4:407–432.

Stambaugh R, Buckley J. Comparative studies of the acrosomal enzymes of rabbit, rhesus monkey, and human spermatozoa. *Biol Reprod* 1970;3:275–282.

Thaler CD, Cardullo RA. Biochemical characterization of a glycosylphosphatidylinositol-linked hyaluronidase on mouse sperm. *Biochemistry* 1995;34:7788–7795.

Thomas P, Meizel S. An influx of extracellular calcium is required for the acrosome reaction induced by human follicular fluid. *Gam Res* 1988;20:397–411.

Towbin HT, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350–4354.

Tung J-S, Mark GE, Hollis GF. A microplate assay for hyaluronidase and hyaluronidase inhibitors. *Analyt Biochem* 1994;223:149–152.

Yudin AI, Cherr GN, Katz DF. Structure of the cumulus matrix and zona pellucida in the golden hamster: a new view of sperm interaction with oocyte-associated extracellular matrices. *Cell Tiss Res* 1988;251:555–564.

19394640, 1997, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/j.1939-4640.1997.tb01895.x by Council of Atlantic Academic Libraries, Wiley Online Library on [20/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License