BIOLOGY OF REPRODUCTION 55, 80–86 (1996)

Sperm Surface Protein PH-20 Is Bifunctional: One Activity Is a Hyaluronidase and a Second, Distinct Activity Is Required in Secondary Sperm-Zona Binding

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ABSTRACT

In previous studies, we have found that the sperm membrane protein PH-20 acts during two different stages of fertilization. On acrosome-intact sperm, PH-20 has a hyaluronidase activity that is required for sperm penetration through the cumulus cell layer that surrounds the oocyte. On acrosome-reacted sperm, PH-20 has a required function in sperm-zona binding (secondary binding). Because hyaluronic acid (HA) has been detected in the zona pellucida, secondary sperm-zona adhesion could depend on repetitive binding and hydrolysis of HA by PH-20 acting as a hyaluronidase. Alternatively, PH-20 may be bifunctional and have a second, different activity required for secondary binding. To distinguish between these two possibilities, in this study we used reagents that inhibit either PH-20's function in sperm-zona binding or its hyaluronidase activity. We found that an anti-PH-20 monoclonal antibody that inhibited sperm-zona binding (~90%) had no effect on hyaluronidase activity. Conversely, apigenin, a hyaluronidase inhibitor, blocked PH-20 hyaluronidase activity 93% without inhibiting sperm-zona binding. Similarly, another anti-PH-20 monoclonal antibody that inhibited hyaluronidase activity 95% only partially inhibited sperm-zona binding (~45%). We also extensively pretreated oocytes with hyaluronidase to remove all accessible HA on or in the zona pellucida and found little or no effect on secondary sperm-zona binding. Our results suggest that PH-20 is bifunctional and has two activities: a hyaluronidase activity and a second, separate activity required for secondary sperm-zona binding.

INTRODUCTION

Fertilization involves numerous interactions between the sperm and the ovulated oocyte. As the sperm approaches the oocyte it initially contacts the cumulus cell layer, a mass of cells surrounding the oocyte. The cumulus layer comprises about 3000 cells held together by an extracellular matrix, and the sperm must penetrate through the cumulus layer to reach the oocyte. Once through the cumulus layer, the sperm encounters the zona pellucida, an extracellular coat encompassing the oocyte. The sperm's initial adhesion to the zona pellucida is through primary binding receptor(s) on the sperm surface, and primary binding induces the sperm to acrosome-react [1–3]. During the acrosome reaction, the membranes surrounding the anterior region of the sperm head are shed, and the acrosomal contents are released into the medium [1]. With the loss of the anterior head membranes and acrosomal contents, a previously internal membrane, the inner acrosomal membrane (IAM), is exposed. The acrosome-reacted sperm binds again to the zona pellucida, through secondary receptor(s) located on the newly exposed IAM [4, 5], and then penetrates the extracellular coat. Once through the zona, the sperm reaches the oocyte plasma membrane, and sperm-oocyte fusion ensues.

PH-20 is a glycosyl phosphatidylinositol (GPI)-anchored membrane protein that can be released from its membrane anchor on the cell surface by treatment with the enzyme PI-PLC [6]. It is located on the posterior head plasma membrane and IAM of guinea pig sperm [4, 7]. When sperm acrosome-react, PH-20 is altered in two major ways: 1) it undergoes proteolytic cleavage, splitting the single 64-kDa polypeptide into two polypeptides (a 41-kDa and a 27-kDa fragment) that are disulfide bonded to each other [8]; and 2) the PH-20 molecules on the posterior head plasma membrane migrate onto the IAM, joining the other population of PH-20 molecules pre-existing on the IAM [9]. Acrosome-reacted sperm binding (secondary binding) to the zona can be inhibited by certain function-blocking anti-PH-20 monoclonal antibodies [4, 5]. Secondary binding is also inhibited by anti-PH-20 polyclonal antibodies, by Fab fragments of these antibodies, and by specific removal of PH-20 from the IAM by treating acrosome-reacted sperm with PI-PLC ([10, 11], unpublished results). These findings indicate that PH-20 has a required role in secondary binding.

Although PH-20 is present on the plasma membrane of acrosome-intact sperm, it is not required in the primary binding of these sperm to the zona pellucida [5]; the function of plasma membrane PH-20 on acrosome-intact sperm has been unknown. Recently we and others found that PH-20 has a hyaluronidase activity in its N-terminal domain [12–14] and that the hyaluronidase activity of plasma membrane PH-20 is required for acrosome-intact sperm to penetrate the cumulus cell layer [14].

Many proteins are now known to be multifunctional and to be composed of modular assemblies of domains that have active sites for the individual functions [15–17]. The finding that PH-20 has required activities at two stages in the process of fertilization suggests that PH-20 could be bifunctional. On the other hand, it is possible that PH-20's required function in secondary binding could depend on the repetitive binding and hydrolysis by its hyaluronidase domain of hyaluronic acid (HA) present in the zona. In this study, we analyzed these two functions further and found that PH-20's two activities are distinct.

MATERIALS AND METHODS

Buffers and Solutions

Media and buffers were as follows. PBS: 10 mM phosphate buffer, 150 mM NaCl at pH 7.4. Calcium Hepes medium: 4 mM Hepes, 140 mM NaCl, 4 mM KCl, 10 mM *d*-glucose, and 2 mM $CaCl_2$, pH 7.4. Oocyte maturation medium: 199 medium (Gibco, Grand Island, NY) supplemented with 20% heat-inactivated fetal bovine serum (Gibco), 10 mM sodium lactate, 1.0 mM pyruvate, 100 U/ml penicillin/streptomycin, and 19 mM $NaHCO_3$, pH 7.4–7.6. Modified Tyrode's medium (MT): 108.8 mM NaCl, 2.8 mM KCl, 0.5 mM $MgCl_2$, 25.1 mM $NaHCO_3$, 5.6 mM *d*-

Accepted February 27, 1996.
Received November 27, 1995.
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glucose, 10 mM sodium lactate, 1.0 mM sodium pyruvate, and 3% BSA, pH 7.4–7.6. PHEM buffer: 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 7.2. Acid albumin buffer: 24 mM sodium acetate, 0.46% glacial acetic acid, and 0.1% BSA, pH 3.75. TNE buffer: 10 mM Tris, 140 mM NaCl, 10 mM EDTA, pH 8.0. The calcium ionophore A23187 was made as a stock solution at 2 mg/ml in dimethyl sulfoxide (DMSO). Lysophosphatidyl choline (LC; Type I; Sigma Chemical Co., St. Louis, MO) was made up fresh as a stock solution at 5 mg/ml in water. Apigenin (Sigma) was made as a stock solution at 25 mM in DMSO.

Antibodies

The antibodies used in this study were three different anti-guinea pig PH-20 monoclonal antibodies—mAb PH-20, mAb PH-21, and mAb PH-22—and a rabbit polyclonal antibody raised against purified guinea pig PH-20. These antibodies have been described previously [4, 18].

Induction of Acrosome Reaction Before Sperm-Zona Binding Assay

LC method: Sperm were removed from regions VI and VII (cauda) of the epididymis as described previously [19, 20], suspended to 10^7 sperm/ml in MT, covered with mineral oil (Squibb, Princeton, NJ), and incubated at 37°C. The sperm were primed to acrosome-react with LC as previously described [5, 21, 22]. Briefly, 35-mm tissue culture plates with the suspended sperm were supplemented with varying concentrations of LC (55, 65, 75, 85, 95, or 105 μg/ml) and incubated for 45–60 min at 37°C. Sperm were induced to acrosome-react by addition of an equal volume of MT supplemented with 4.0 mM calcium to give a final calcium concentration of 2.0 mM and were incubated an additional 30 min at 37°C. The plate with the highest percentage of acrosome-reacted sperm showing hyperactive motility was used as the acrosome-reacted sperm source in the sperm-zona binding assay.

Oocyte Collection

Ovaries from young (250–350-g) guinea pigs were removed and placed in oocyte maturation medium [5, 23]. Oocytes were released from the ovary by poking follicles with a 30-gauge needle and were mouth-pipetted through 5 wash drops (200 μl) of oocyte maturation medium. The oocytes were placed in a final oocyte maturation medium drop (200 μl), covered with mineral oil, and incubated overnight in a 37°C, 5% CO_2 humidified incubator. The cumulus was removed by gently mouth-pipetting the oocytes through a pipette with a bore equal to the diameter of the zona-intact oocyte (in order to obtain cumulus-free oocytes that had not been exposed to exogenous hyaluronidase). The oocytes were pipetted through 5 wash drops (200 μl each) of MT, supplemented with 2.0 mM calcium, and placed in a final droplet of 200 μl MT with calcium, covered with mineral oil, and returned to the incubator until ready to use.

Hyaluronidase Treatment of Oocytes

Guinea pig oocytes, mechanically freed of cumulus cells, were incubated with a hyaluronidase (purified, recombinant, cynomolgus monkey PH-20, lacking the GPI anchor so that it is soluble [14, 24]) at 67 000 U/ml in MT medium at 37°C for 3.5 h. Oocytes were washed and placed into fresh MT medium, and the sperm-zona binding assay carried out as usual. Control oocytes (no treatment) were incubated and washed in MT medium in parallel.

Sperm-Zona Binding Assay

Basic assay. Oocytes were transferred to a 90-μl, 37°C droplet of MT supplemented with 2 mM calcium. Approximately 10^5 acrosome-reacted sperm (10 μl of the LC acrosome-reacted sperm) were added to the droplet and incubated for 20–30 min. The oocytes and bound sperm were gently pipetted away from the incubation drop and into a 500-μl wash droplet of MT supplemented with calcium. After 5 min, sperm and oocytes were fixed by adding 100 μl of 12% formaldehyde to the wash droplet (giving a 2% formaldehyde final concentration). Oocytes were transferred to a microscope slide and examined without being immobilized. Sperm bound to the oocyte's zona pellucida were counted in a standard plane of focus that passed approximately through the center of the oocyte.

Apigenin treatment. The suspension of LC acrosome-reacted sperm was split into two equal samples. Apigenin (from a 25 mM stock solution in DMSO) was added to one sample to a final concentration of 250 μM, while DMSO was added to the other sample to final concentration of 1% (for a solvent control). The sperm were preincubated with these additions for 15–30 min before they were added to oocytes. Oocytes were transferred to a 90-μl, 37°C droplet of MT supplemented with 2 mM calcium and either 250 μM apigenin or 1% DMSO. The apigenin-treated and DMSO-treated sperm were added to their corresponding droplets containing oocytes. Sperm bound per zona pellucida were counted as above. The hyaluronidase activity was determined for samples of the apigenin-treated and DMSO-treated sperm used in the sperm-zona binding assay.

Affinity Purification of PH-20 Protein

Sperm (from five guinea pigs) were removed from regions VI and VII (cauda) of the epididymis as described previously [19, 20], suspended in calcium Hepes at 10^7 sperm/ml, and induced to acrosome-react by use of the calcium ionophore A23187 at 2 μg/ml as previously described [9]. The acrosome-reacted sperm were pelleted by centrifugation (750 × *g* for 10 min at room temperature) and were resuspended in PHEM buffer. PH-20 was released from the sperm membrane with PI-PLC: the acrosome-reacted sperm were washed through PHEM buffer twice and resuspended in 5 ml PHEM buffer (~4×10^8 sperm/ml) and 0.25 U of PI-PLC (PI-PLC from *Bacillus cereus*, spec. act. 600 U/mg, catalog #1143069; Boehringer Mannheim, Indianapolis, IN). The tube was covered with aluminum foil to protect the light-sensitive enzyme and placed on a rocker at 37°C for 60 min. Sperm remnants and particulates were pelleted by ultra centrifugation at 150 000 × *g* for 45 min at 4°C (Beckman TLA-100.3 rotor, 60 000 rpm; Beckman Instruments, Palo Alto, CA). The upper 3/4 of the supernatant was carefully removed with a Pasteur pipette, and this supernatant was dialyzed against TNE buffer. The dialyzed material was passed over a precolumn of Sepharose CL-4B (Sigma), the flow through was collected and loaded onto an affinity column (mAb PH-22 cross-linked Sepharose), and after washing, the bound material was eluted with diethylamine (pH 11.5) as previously described [8].

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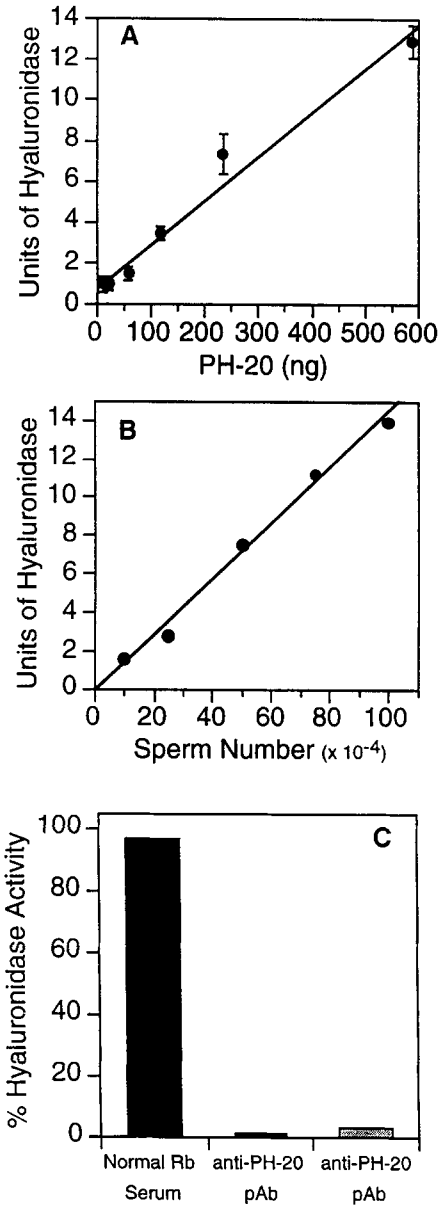

FIG. 1. Hyaluronidase activity of purified and surface bound PH-20. **A**) Increasing amounts of purified PH-20 from acrosome-reacted sperm were assayed for hyaluronidase activity and compared to standard curve of activity that was run in parallel. Data points were taken in triplicate; error bars represent standard deviation. **B**) Guinea pig sperm were acrosome-reacted with calcium ionophore A23187, washed, and counted. Increasing amounts of acrosome-reacted sperm were assayed for hyaluronidase activity and compared to standard curve of activity that was run in parallel. Number of sperm used for data points is in range 10×10^4-100×10^4. Graph is of representative experiment. **C**) Affinity-purified PH-20 (200 ng) from acrosome-reacted sperm (black bars) and 3×10^6 acrosome-reacted guinea pig sperm (grey bar) were preincubated with 1:500 dilution of rabbit anti-guinea pig PH-20 antiserum for 30 min at 37°C. Samples were tested for hyaluronidase activity and are shown as percentage of no-antibody control. Dilution (1:500) of normal rabbit serum was used as control with purified PH-20.

Hyaluronidase Activity Assay

The assay used was a modification of the spectrophotometric, turbidity-clearing protocol of Dorfman [25]. Briefly, various concentrations of affinity-purified, PI-PLC-released PH-20 from acrosome-reacted sperm or various numbers of whole, acrosome-reacted sperm were placed in 0.5 ml of PBS (pH 7.4) supplemented with 0.01% BSA and were mixed with 0.5 ml of a 0.15 mg/ml solution of HA in PBS for a substrate. The enzymatic digestion of the HA was carried out at 37°C for 45 min. Undigested HA was precipitated at the end of the incubation by addition of 5 ml of acid albumin buffer. The degree of turbidity of each sample was determined by measuring the OD_{600nm} 5 min after the addition of the acid albumin buffer. The spectrophotometer was blanked against a PBS-BSA, acid albumin control. The units of activity for each sample were determined by comparing the OD_{600nm} of the sample to the OD_{600nm} of a standard curve of hyaluronidase activity, generated from a solution of bovine testicular hyaluronidase with a specific activity of 290 U/mg (Sigma H-3506).

RESULTS

Membrane-Bound PH-20 on Acrosome-Reacted Guinea Pig Sperm Has Hyaluronidase Activity

It has previously been found that recombinant mouse, monkey, and human PH-20 have hyaluronidase activity [13, 14]. To determine whether a hyaluronidase activity of PH-20 is the activity required in secondary sperm-zona binding in guinea pigs, we first confirmed that guinea pig PH-20, purified from acrosome-reacted cells, has hyaluronidase activity. We affinity-purified PH-20 from acrosome-reacted sperm [8] and determined hyaluronidase activity by use of a turbidimetric assay [25]. The purified PH-20 showed hyaluronidase activity (Fig. 1A).

To show that PH-20 while anchored on the IAM has hyaluronidase activity, we measured enzyme activity on acrosome-reacted sperm cells. Before assaying, sperm were extensively washed to remove loosely associated, soluble hyaluronidase from the acrosomal contents. The washed acrosome-reacted sperm showed high levels of hyaluronidase activity (Fig 1B). An anti-PH-20 polyclonal antibody that inhibited hyaluronidase activity of the purified PH-20 (Fig. 1C) also inhibited hyaluronidase activity on acrosome-reacted sperm 95% (Fig 1C), indicating that PH-20 is the source of the surface hyaluronidase of acrosome-reacted cells.

Apigenin Inhibits Sperm Surface Hyaluronidase Activity but Not Sperm-Zona Binding

To determine whether the surface hyaluronidase activity of acrosome-reacted sperm was required for secondary sperm-zona binding, apigenin, an inhibitor of PH-20 hyaluronidase activity [14], was tested as an inhibitor of secondary binding. An aliquot of acrosome-reacted sperm tested in the presence of 250 μM apigenin showed a 93% inhibition of the surface hyaluronidase activity (Fig. 2A). When another aliquot of the same acrosome-reacted sperm preparation was added to oocytes in the continuing presence of 250 μM apigenin, we saw no reduction in the level of sperm-zona binding between the apigenin-treated sample and controls (Fig. 2B).

mAb PH-20 Inhibits Sperm-Zona Binding but Not Sperm Hyaluronidase Activity

In previous studies (and in this study, see Fig. 4), we found that one function-blocking monoclonal antibody, mAb PH-20, inhibits secondary sperm-zona binding ~90%. A different, nonfunction-blocking monoclonal antibody to PH-20, mAb PH-22, has no effect on sperm-zona binding [4]. Thus, mAb PH-20 binds so as to inhibit the active site in PH-20 for secondary sperm-zona adhesion. We therefore

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investigated whether mAb PH-20 would also inhibit hyaluronidase activity. Acrosome-reacted sperm were preincubated with mAb PH-20 and assayed for hyaluronidase activity (Fig. 3). We saw no significant inhibition of hyaluronidase activity on mAb PH-20-treated sperm. Likewise, only very slight inhibition of hyaluronidase was detected on sperm treated with mAb PH-22 (Fig. 3). Interestingly, a third monoclonal antibody to PH-20 (mAb PH-21), which shows a partial inhibitory effect (~45%) in secondary sperm-zona binding studies [4], inhibited the hyaluronidase activity of acrosome-reacted sperm 95% (Fig. 3).

Sperm Still Bind to Oocytes Extensively Treated with Hyaluronidase

Using another approach to determine whether HA associated with the zona pellucida is involved in secondary sperm-zona binding, we extensively treated zona-intact oocytes with a hyaluronidase to hydrolyze accessible HA. The source of hyaluronidase we used was recombinant, purified cynomolgus monkey PH-20 [14, 24]. Zona-intact oocytes were treated with 67 000 U/ml of hyaluronidase for 3.5 h at 37°C. (For comparison, the cumulus layer is typically removed from oocytes using 725 U/ml of hyaluronidase at 37°C for 4–5 min [5]). LC acrosome-reacted sperm were mixed with these oocytes, and 20–30 min later the number of sperm bound to the zonae were counted. We saw little or no difference in sperm binding to the zonae between the hyaluronidase-treated oocytes and the controls (Table 1).

We also investigated whether the observed binding of acrosome-reacted sperm to hyaluronidase-treated oocytes still involved PH-20 and was not the result of some other, functionally redundant mode of binding. In these experiments, we determined the effect of mAb PH-20 on the binding of acrosome-reacted sperm to the zona of oocytes extensively treated with hyaluronidase. Monoclonal antibody PH-20 still strongly inhibited sperm-zona binding under these conditions (Fig. 4).

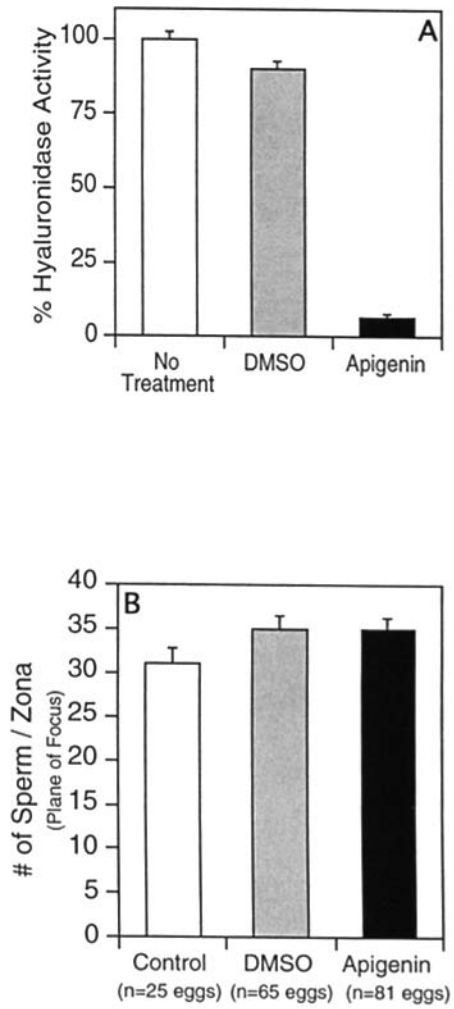

FIG. 2. Effects of apigenin on hyaluronidase activity of acrosome-reacted sperm and on sperm-zona binding assay. **A**) LC-induced, acrosome-reacted sperm were assayed for hyaluronidase activity. Approximately 10^6 sperm were assayed in presence of 250 μM apigenin and 1% DMSO (solvent), in presence of 1% DMSO alone, and with no treatment. Values are plotted as percentage of activity compared to no-treatment control. Error bars represent SEM. **B**) Aliquot of 10^4 sperm from sperm preparation tested in **A** was incubated with 250 μM apigenin/1% DMSO in MT medium for 30 min before being mixed with zona-intact guinea pig oocytes, also in 250 μM apigenin/MT medium. Sperm and oocytes were incubated together at 37°C for 20–30 min before being fixed in 2% formaldehyde. Data shown are from single experiment that was repeated with equivalent results. Sperm bound to zona in single plane of focus were counted. No-treatment and 1% DMSO-treated samples were run as controls. Error bars represent SEM. Number of oocytes examined is given in parentheses.

DISCUSSION

In previous work, we have made two findings about PH-20: it has a hyaluronidase activity and a required function in secondary sperm-zona binding (see *Introduction*). These results suggest two hypotheses. First, the required function of PH-20 in secondary binding could be a manifestation of its hyaluronidase activity if secondary sperm-zona binding depends on multiple and continuous rounds of binding and hydrolysis of HA in the zona. The alternative hypothesis is that PH-20 is a bifunctional protein with two distinct activities, one as a hyaluronidase, the other in secondary binding. The experiments in this study were done to evaluate these hypotheses.

One approach we used was to test reagents that block either the function of PH-20 in sperm-zona binding or its hyaluronidase activity. We found that mAb PH-20, which blocks secondary sperm-zona binding, does not inhibit PH-20's hyaluronidase activity. This result suggests that the function in secondary binding and the hyaluronidase function are two separate activities. We also obtained the converse result that PH-20's hyaluronidase activity could be blocked 93% (by apigenin) without inhibiting secondary sperm-zona binding. This indicates that hyaluronidase activity is not needed for secondary binding and that the PH-20 protein has two distinct activities. The results with mAb PH-20 and apigenin suggest that the hyaluronidase active site and the active site for secondary binding could be spatially separated in PH-20. The partial (45%) inhibition of secondary binding by mAb PH-21 that blocks (> 95%) hyaluronidase activity may mean that the PH-21 epitope is in the hyaluronidase active site, yet antibody binding also partially interferes with access to a separate PH-20 site involved in secondary binding.

A different approach was to remove the putative HA substrate in the zona pellucida by extensively pre-treating the oocytes with a hyaluronidase. Because we found that commercial hyaluronidase preparations are impure and at least in one case contaminated with proteases, we used purified, recombinant monkey PH-20 as the source of hyaluronidase activity [14]. HA has been identified in the zona through use of nonquantitative, morphological staining techniques [26]. We are not aware of chemical techniques

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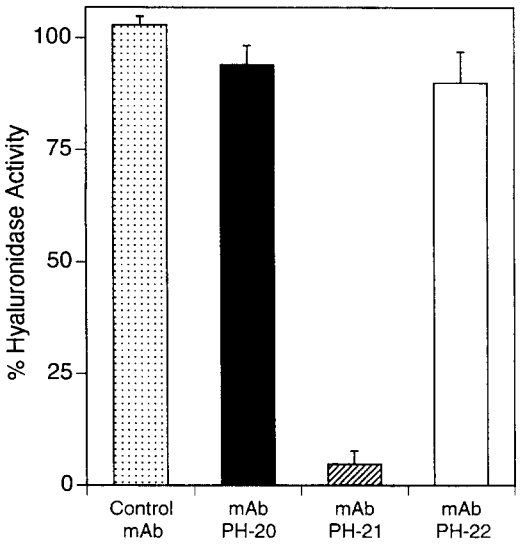

FIG. 3. Effect of three different anti-PH-20 monoclonal antibodies on hyaluronidase activity of acrosome-reacted sperm. A23187 ionophore-induced, acrosome-reacted sperm were pretreated with either mAb PH-20, mAb PH-21, or mAb PH-22 for 30 min at 37°C. Sperm were washed free of unbound antibody and assayed for hyaluronidase activity. Irrelevant antisperm monoclonal antibody (mAb PH-30) was used as control. Activity is expressed as percentage of no-treatment control. Error bars represent SEM.

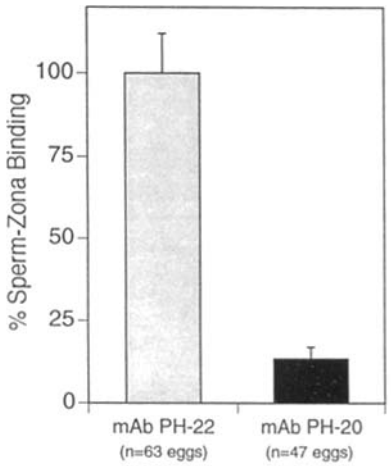

FIG. 4. Inhibition by mAb PH-20 of sperm binding to zona-intact oocytes extensively pretreated with hyaluronidase. Zona intact oocytes were pretreated with hyaluronidase (as in Table 1) and washed. LC-induced, acrosome-reacted sperm were preincubated with 150 μg/ml of mAb. Sperm and oocytes were mixed and incubated together for 20 min at 37°C before being fixed in 2% formaldehyde, and sperm bound to zona in single plane of focus counted. Sperm were preincubated with mAb PH-22 as control; binding is expressed as percentage of this control. Number of oocytes examined is given in parentheses. Error bars represent SEM.

sensitive enough to allow quantification of the amounts of zona-associated HA before and after hyaluronidase treatment. Thus, we cannot rule out that some HA in the zona was inaccessible or, for other reasons, not subject to enzymatic hydrolysis. However, the zona is readily accessible to proteins (purified PH-20 in this case), and our conditions were designed to maximize HA degradation. For sperm-zona binding assays, oocytes are typically pretreated with hyaluronidase at 725 U/ml for 4–5 min at 37°C to remove the cumulus layer. For our extensive hyaluronidase digestion, we treated oocytes (whose cumulus layer had been removed mechanically) with 67 000 U/ml hyaluronidase for 3.5 h at 37°C. Despite this extensive pretreatment with hyaluronidase, no reduction in sperm-zona binding was observed. Also, sperm binding remained inhibitable by mAb PH-20, indicating that hyaluronidase treatment had not created a new mode of sperm binding, while abolishing PH-20's role in adhesion.

Collectively, the experiments in this study support the conclusion that PH-20 has two distinct activities, a hyaluronidase activity and a different activity required in secondary adhesion. Because the two activities of PH-20 are apparently separate, the two active sites may be located in distinct domains of the protein. During the acrosome reaction, proteolytic cleavage occurs between residues 311 and 312, i.e., between the C-terminal domain and the N-terminal domain, creating a protein with two disulfide-bonded subunits [8] (Fig. 5). The hyaluronidase active site is presumably in the PH-20 N-terminal region (41 kDa on SDS-PAGE, residues 1–311), since amino acids 17–307 have sequence homology with bee venom hyaluronidase [12]. It is possible that the active site involved in secondary sperm-zona binding is in the C-terminal region (27 kDa on SDS-PAGE, residues 312–468 [8]) or at the interface between the two domains (Fig. 5). Proteolytic cleavage produces a new N-terminus (at amino acid 312), and many

TABLE 1. Sperm binding to zona-intact oocytes extensively pretreated with a purified hyaluronidase.

Experiment	Treatment	No. of sperm bound/oocyte (No. of oocytes tested)	% Control
1	Control	22.2 ± 8.7 (26)	(100)
	Hyaluronidase	21.9 ± 7.7 (38)	99
2	Control	30.2 ± 9.1 (45)	(100)
	Hyaluronidase	26.6 ± 9.7 (73)	88

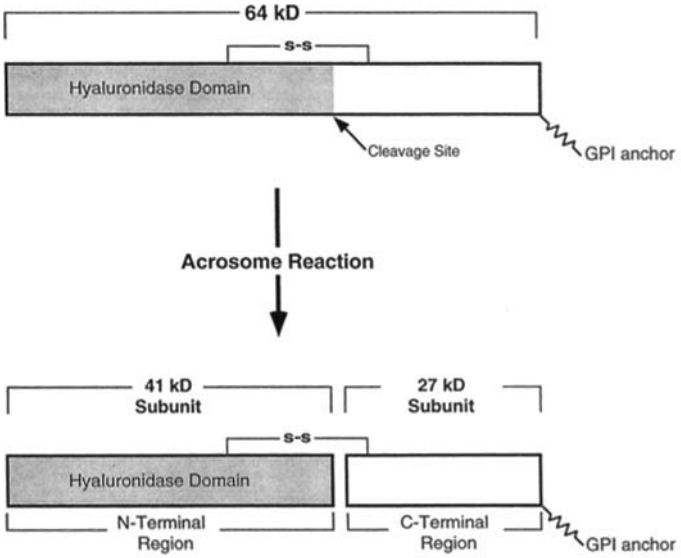

FIG. 5. Diagram of guinea pig PH-20 before and after acrosome reaction. PH-20, on acrosome-intact guinea pig sperm, is GPI-anchored, single polypeptide of ~64 kDa, 468 amino acids long ([8, 18], unpublished results). Portion of molecule with sequence homology to bee venom hyaluronidase (shaded area) is located at N-terminal region ending at amino acid 307 [12]. Upon acrosome reaction, PH-20 is cleaved into two polypeptides (41-kDa fragment and 27-kDa fragment) that are held together by disulfide bond(s) [8]. Site of cleavage is between amino acids 311 (arginine) and 312 (serine) [18] (unpublished results).

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adhesion proteins bind to their ligand near their N-terminus [27–36]. Thus, cleavage may be essential in activating a function of the C-terminal domain.

The data showing that mAb PH-20 blocks secondary binding and mAb PH-21 blocks hyaluronidase activity suggest that each monoclonal antibody may bind near (or at) a corresponding active site. The location of the two active sites might be defined more precisely by mapping the epitopes recognized by mAb PH-20 and mAb PH-21. Current attempts in mapping these epitopes, however, have been unsuccessful; neither of the monoclonal antibodies works in immunoblots, and we have not been able to separate the two subunits in native form by standard methods.

If the required activity of PH-20 in secondary binding is distinct from its hyaluronidase activity, what might this required activity be? Secondary binding of sperm to the zona is unusual among cell adhesion phenomena in that it is transient and is shortly followed by sperm release that must occur to allow penetration of the zona. It is not known whether some amount of sperm penetration of the zona occurs before secondary binding is initiated. Because secondary binding is poorly understood, it may be hard to guess what the required activity of PH-20 might be. One possibility is that PH-20 has an adhesion activity. The C-terminal domain of PH-20 does not have homology to known adhesion proteins. This, however, is consistent with the fact that each of the zona proteins, the potential ligand(s) for any sperm adhesion protein, is specific to gametes and apparently unrelated to proteins of other cell types. We have tested to see if purified, cleaved PH-20 or heterologous cell types expressing uncleaved PH-20 (or PH-20 cleaved by brief trypsin addition) will bind to the zona. These experiments have so far given negative results. Thus, there is no evidence at this time that the required function of PH-20 in secondary binding is an adhesion activity, but many tests of this hypothesis remain to be done.

The results presented here may be an example of a common finding that proteins comprised separate domains with separate functions [15–17, 37]. Indeed, the ability of cells to make such proteins is of fundamental importance in the course of evolution [15]. The evolution of PH-20 as a bifunctional protein may reflect the unusual demands that sperm meet in penetrating through and binding to the various layers surrounding the oocyte plasma membrane.

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