Journal of Andrology, Vol. 23, No. 2, March/April 2002

Importance of Glycosylation and Disulfide Bonds in Hyaluronidase Activity of Macaque Sperm Surface PH-20

MING-WEN LI,* ASHLEY I. YUDIN,* KATHRYN R. ROBERTSON,* GARY N. CHERR,† AND JAMES W. OVERSTREET*

*From the *Division of Reproductive Biology, Department of Obstetrics and Gynecology, and †Bodega Marine Laboratory and Departments of Environmental Toxicology and Nutrition, University of California, Davis, California.*

ABSTRACT: PH-20 is a glycoprotein located on the surface of the sperm plasma membrane and on the inner acrosomal membrane. The best understood function of sperm surface PH-20 is its hyaluronidase activity, which results in hydrolysis of the hyaluronic acid–rich cumulus matrix during sperm penetration of this extracellular oocyte investment. In this study, we investigated whether alterations in the secondary and tertiary structures of sperm surface PH-20 would affect its enzyme activity. Proteins were isolated from the sperm plasma membrane by treatment of living cells with phosphatidylinositol-specific phospholipase C (PI-PLC). PH-20 was purified from the PI-PLC released proteins by immunoaffinity chromatography. Two-dimensional electrophoresis of purified PH-20 revealed 6 isoforms with isoelectric points ranging from 5.1 to 6.0. Removal of the N-linked glycans from PH-20 with N-glycosidase F shifted the molecular weight from 64 kd to approximately 54 kd, its deduced molecular weight based on sequence analysis, suggesting that most if not all, of the potential N-glycosylation sites are linked to oligosaccharides. The lectins Con A and PSA recognized purified sperm surface PH-20 after Western blotting, suggesting that mannose is a major sugar within or at the terminal end of the linked glycan. The lectins UEA and LPA did not recognize PH-20 Western blot, suggesting that fucose and sialic acid are not terminal sugars of sperm surface PH-20. Deglycosylation of sperm surface PH-20 resulted in a complete loss of its hyaluronidase activity. The reduction of disulfide bonds with β-mercaptoethanol or dithiothreitol also resulted in loss of enzyme activity. We conclude that the hyaluronidase activity of sperm surface PH-20 is dependent on structural features established by sulfhydryl linkages, as well as glycosylation.

Key words: Enzyme activity, glycoprotein, protein structure, deglycosylation, lectins.

J Androl 2002;23:211–219

PH-20 is a glycosylphosphatidylinositol-anchored glycoprotein present on the plasma membrane of mammalian sperm heads and on the inner acrosomal membrane (Myles and Primakoff, 1984; Lin et al, 1994; Overstreet et al, 1995). Mammalian sperm are highly compartmentalized, and have numerous membrane domains (Bearer and Friend, 1990). The functions of PH-20 are varied and are related to its various locations in sperm. Initially, PH-20 on the inner acrosomal membrane of guinea pig sperm was recognized as a binding ligand for the zona pellucida (Myles et al, 1987a). Later, PH-20 was found to be the sperm hyaluronidase (Gmachl and Kreil, 1993). PH-20 found on the plasma membrane and inner acrosomal membrane has hyaluronidase activity, as does a soluble form of the protein, which is believed to be processed from PH-20 on the inner acrosomal membrane (Cherr et al, 1996; Li et al, 1997). The enzyme action of plasma membrane PH-20 has been shown to facilitate sperm penetration of the cumulus oophorus (Lin et al, 1994; Cherr et al, 1996). In addition, plasma membrane PH-20 interacts with hyaluronic acid in the cumulus matrix to raise the internal concentration of calcium in sperm, an action that may ''prime'' the sperm for induction of the acrosome reaction by zona pellucida proteins (Sabeur et al, 1997; Cherr et al, 1999). In light of the numerous fertilization-related functions of PH-20, it is not surprising that female guinea pigs became infertile after they were immunized against PH-20 (Primakoff et al, 1988b).

The various actions of a multifunctional glycoprotein may be regulated by its secondary and tertiary structures. PH-20 is known to have a number of potential N-glycosylation sites, and as many as 12 cysteines are conserved in the PH-20 of different mammalian species (Lin et al, 1993). Addition of oligosaccharides (glycans) to protein structures is a common occurrence in higher organisms (Varki and Freeze, 1994). These secondary gene products can be species specific, tissue specific, cell specific, or a combination of these (Kobata, 1992). One of the most important functions of N-linked glycans involves the regulation of protein conformation and the stabilization of intramolecular folding, which is critical for biological ac-

Supported in part by grants U54-HD29125 and P51-RR00169 from the National Institutes of Health, and by the Andrew W. Mellon Foundation.

Correspondence to: James W. Overstreet, MD, PhD, Institute of Toxicology and Environmental Health, University of California, Davis, One Shields Avenue, Davis, CA 95616

Received for publication June 19, 2001; accepted for publication September 12, 2001.

tivity and protection (Lis and Sharon, 1993; Varki, 1993; Zhang et al, 1995). Glycosylation is a common characteristic of cell surface proteins, and variation in protein glycosylation is responsible for diversity of biological functions in protein isoforms (Gagneux and Varki, 1999; Apweiler et al, 1999).

In this manuscript we report the results of studies designed to investigate the secondary and tertiary structures of macaque sperm PH-20 and how variation in these structures is related to the biological functions of the protein. We report the presence of a number of isoforms of sperm surface PH-20, and we use lectins to evaluate the diversity of carbohydrates associated with the PH-20 molecule. We also determined whether glycosylation was necessary for the hyaluronidase activity of PH-20. Because the cysteines are conserved within all hyaluronidases, we propose that intramolecular folding due to sulfhydryl linkages is required for hyaluronidase activity.

Materials and Methods

Chemicals

Biotinylated lectin kits I, II, and III, and alkaline phosphatase-labeled anti-biotin antibodies were purchased from Vector Laboratories (Burlingame, Calif). After screening, the following lectins were used: concanavalin A (Con A) isolated from seeds of the Jack bean, *Canavalia ensiformis*; *Pisum sativum* agglutinin (PSA) isolated from seeds of the garden pea, *P sativum; Phaseolus vulgaris* agglutinin with 4 ''E'' subunits (PHA-E) isolated from seeds of the red kidney bean, *P vulgaris*; Jacalin, isolated from seeds of the Jackfruit, *Artocarpus integrifolia*; *Lycopersicon esculentum* lectin (LEL) isolated from tomato fruit; and *Solanum tuberosum* lectin (STL) isolated from potato tubers. Chemicals used for electrophoresis and Western blotting were purchased from Bio-Rad Laboratories (Hercules, Calif). All other chemicals and reagents were purchased from Sigma Chemical Company (St Louis, Mo) unless stated otherwise.

Sperm Collection and Processing

Semen samples were collected by electroejaculation from 10 individually caged cynomolgus macaques (Sarason et al, 1991). Animals were housed at the California Regional Primate Research Center in compliance with the Federal Animal Welfare Act and the National Institutes of Health *Guidelines for Care and Use of Laboratory Animals.* Each ejaculate was collected into a 15-mL centrifuge tube containing 5 mL of HEPES-buffered Biggers Whitten Whittingham (BWW) medium (Irvine Scientific, Santa Ana, Calif). After liquefaction of the semen at room temperature for 1 hour, the top 4 mL of the sperm suspension were transferred to another tube and centrifuged for 10 minutes at 300 × *g.* Sperm pellets were resuspended in 2 mL of Dulbecco phosphate-buffered saline (DPBS, Life Technologies, Rockville, Md) and layered over 4 mL of 40% Percoll in DPBS and centrifuged at 300 × *g* for 15 minutes. Then, the sperm were washed an additional 2 times in 10 mL of DPBS with centrifugation at 300 × *g.*

Treatment of Sperm to Recover Sperm Surface PH-20

Washed sperm pellets were resuspended at a concentration of 100 million/mL in DPBS containing 3 units/mL of phosphatidylinositol-specific phospholipase C (PI-PLC) and a mixture of protease inhibitors (20 mM EDTA, 1 mM p-hydroxymercurobenzenzoate, 5 mM N-ethymalemide, and 1 mM benzamidine). Following 2 hours of incubation at 37°C with gentle rolling, the sperm samples were centrifuged at 1000 × *g* for 10 minutes, and the supernatant was passed through a 0.22-μm syringe filter. The supernatants were transferred to another tube and ultracentrifuged for 1 hour. The PI-PLC released proteins were concentrated threefold at 4°C using a Centricon 10 ultrafiltration tube (Millipore, Bedford, Mass), and were stored at −80°C. The PI-PLC released proteins were subsequently separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed as described below. PH-20 protein was purified from PI-PLC released proteins by immunoaffinity chromatography as described below.

Purification of PH-20

The antibody designated 4639 was prepared by purifying PI-PLC released plasma membrane PH-20 (PM-PH-20) on an R-10 immunoaffinity column. The R-10 immunoglobulin (Ig) G was an antibody developed to the recombinant cynomolgus PH-20 (Overstreet et al, 1995). PM-PH-20 was used as an antigen in rabbits, and the subsequent immunoglobulin was isolated on a protein A column. Immunoaffinity beads were prepared by coupling rabbit anti-PH-20 IgG (4639) to Actigel-ALD resin (Sterogene, Carlsbad, Calif) according to the manufacturer's instructions. Briefly, 5 mg IgG in 2.5-mL coupling buffer (0.1 M Na_2HPO_{4}-NaH_2PO_4 pH 8.0) and 0.5 mL of ALD-coupling solution were added to 2.5 mL of washed Actigel-ALD resin, and incubated for 3 hours at room temperature. The resin was then washed with 30 mL of 0.5 M NaCl; endcapped by adding 6 mL coupling buffer, 1 mL ALD-coupling solution, and 50 μL ethandamine; and incubated for 2 hours at room temperature. To purify PH-20, the affinity beads were pre-equilibrated with TTBS (50 mM Tris-HCl pH 7.5, containing 0.025% Tween 20, 0.025% Triton X-100, and 0.3 M NaCl) and then incubated with PI-PLC released sperm surface proteins for 3–5 hours at 8°C in the presence of 0.05% Tween 20. The beads were centrifuged to remove the supernatant and washed 5 times (30 gel volumes) with TTBS. PH-20 was eluted by incubation with 2–3 gel volumes of elution buffer (50 mM glycine-HCl pH 3.0) for 5 minutes with rolling. The eluted PH-20 was neutralized immediately by addition of 0.1 volume of 1 M Tris-HCl (pH 8.0). PH-20 was concentrated using Centricon 10 ultrafiltration tubes, and stored at −80°C prior to use. PH-20 concentration was measured using BCA protein assay regents (Pierce, Rockford, Ill).

Deglycosylation or Reduction of PH-20

To remove N-linked glycans from PH-20, approximately 5 μg of immunoaffinity-purified PH-20 protein was treated with 10 units/0.1 mL N-glycosidase F (Boehringer-Manneheim GmbH, Mannheim, Germany) in 100 mM Tris-HCl pH 7.5, in the pres-

ence of 0.1 % SDS, 0.5% Triton X-100, 0.5% n-octyl β-D-glucopyranoside, and a mixture of protease inhibitors (0.04 mg/mL phenylmethylsulfonyl fluoride, 20 mM EDTA, 1 mM p-hydroxymercurobenzenzoate, 5 mM N-ethymalemide, and 1 mM benzamidine) at 37°C overnight. To remove possible O-linked glycans from PH-20, approximately 5 μg of purified PH-20 protein was treated with 5 mU/0.1 mL O-glycosidase (Roche Diagnostics GmbH, Germany) in DPBS pH 7.2, in the presence of 0.05% SDS and 1% Triton X-100 at 37°C overnight. Both glycosidases were used according to the manufacturer's specifications. Controls consisted of PH-20 proteins treated in an identical manner but without N-glycosidase F or O-glycosidase. The deglycosylated protein was then used for Western blotting or hyaluronic acid substrate gel assay. To determine the function of disulfide bonds in PH-20 hyaluronidase activity, samples of purified PH-20 were incubated at 37°C for 15 minutes in SDS sample buffer in the absence (control) or presence of 5% β-mercaptoethanol or 100 mM dithiothreitol (DTT) before the hyaluronic acid substrate gel assay.

SDS-PAGE and Western Blotting

Electrophoresis of the surface proteins of PI-PLC treated sperm was carried out using precast 10% or 4%–20% Tris-glycine gels (Fisher Scientific, Pittsburgh, Pa). Approximately 3–5 μg of sperm protein were loaded onto each well. After SDS-PAGE, some gels were stained with GelCode Blue protein stain or GelCode glycoprotein stain. For other gels, proteins separated by SDS-PAGE were electrotransferred to nitrocellulose membranes (100 V for 1 hour at 4 °C). Then, the nitrocellulose membranes were blocked by incubation overnight in TTBS (50 mM Tris-HCl, 0.3 M NaCl, 0.02% Tween 20 pH 7.5) containing 5% nonfat dry milk and 0.1% NaN_3 at room temperature, and probed either with rabbit IgG raised against sperm surface PH-20 (4639) (Lin et al, 1994), or against Peptide 1, a synthesized peptide representing a region of the PH-20 molecule (Yudin et al, 2001), or with control preimmune IgG for 2 hours at room temperature. After being thoroughly washed, the nitrocellulose membrane was incubated for 1 hour in alkaline phosphatase-labeled goat anti-rabbit IgG (Bio-Rad) at a concentration of 1:3000 in TTBS containing 3% BSA and 0.1%NaN_3. The membrane was washed thoroughly in TTBS and the immune complexes were detected using precipitating alkaline phosphatase substrate (1-step NBT-BCIP, Pierce).

Lectin Blotting

Following the procedures for SDS-PAGE and Western blotting, the nitrocellulose membrane was blocked in 3% bovine serum albumin (BSA) in TTBS at room temperature overnight. The blot was then incubated for 2 hours with biotinylated lectin at a concentration of 1 μg/mL in TTBS containing 3% BSA and 10 mM Ca^{2+} and 10 mM Mn^{2+} at room temperature. After being washed thoroughly with TTBS and incubated in alkaline phosphatase-labeled anti-biotin antibodies for 1 hour, the membrane was washed again in TTBS and developed in 1-step NBT-BCIP (Pierce).

Two-Dimensional PAGE

Two-dimensional electrophoresis was performed using Mini-PROTEIN II 2-D Cell (Bio-Rad) according to the manufacturer's instructions. The first dimension (isoelectric focusing; IEF) gel monomer solution contained 9.2 M urea, 4% acrylamide, 20% Triton X-100, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, 0.01% ammonium persulfate, and 0.1% N,N,N′,N′-Tetramethylethylenediamine (TEMED). The upper chamber buffer was 20 mM NaOH, and the lower chamber buffer was 10 mM H_3PO_4. The IEF dimension gels were pre-electrophoresed at 200 V for 10 minutes, 300 V for 15 minutes, and 400 V for 15 minutes. Then, 20 μL of sample containing 1 part of first-dimension sample buffer (9.5 M urea, 2.0% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-lyte 5/7 ampholyte, and 0.4% Bio-Lyte 3/10 ampholyte) and 1 part of PH-20 sample (~5 μg PLC-released sperm surface proteins) were loaded onto the top of each tube gel, and then overlaid with 10 μL of overlay buffer (9 M urea, 0.8% Bio-Lyte 5/7 ampholyte, 0.2% Bio-Lyte 3/10 ampholyte). The gels were run at 400 V for 10–13 hours. The second dimension SDS-PAGE was performed by loading extruded tube gel horizontally onto the top of discontinuous polyacrylamide slab gel (10% separating gel and 4% stacking gel). The proteins separated by SDS-PAGE were electrotransferred to nitrocellulose membranes (100 V for 1 hour at 4°C) and stained with BLOT-FastStain (Chemicon International Inc, Temecula, Calif). Two-dimensional SDS-PAGE standards were used to determine the isoelectric points of PH-20 isoforms. Nitrocellulose membranes were then destained in warm deionized water and blocked in 3% BSA before immunoblotting to detect PH-20. SeeBlue prestained protein standards (Novex, San Diego, Calif) were used to determine the molecular weights of separated proteins.

Hyaluronic Acid Substrate Gel Assay

PH-20 hyaluronidase activity was assessed by hyaluronic acid substrate gel electrophoresis (Li et al, 1997). Briefly, samples were electrophoresed on 7.5% SDS polyacrylamide gels containing hyaluronic acid, and after removal of SDS, the gels were incubated at pH 7.0 for 15 to 20 hours at 37°C. Gels were stained with Alcian blue and counterstained with Coomassie blue to detect areas of hyaluronic acid digestion. Prestained protein standards, SeeBlue, were used to determine the hyaluronidase molecular weights.

Results

A number of proteins were released from the sperm surface after treatment of sperm with PI-PLC (Figure 1B). The 64-kd band, as well as a few others (14 kd, 24 kd, and 53 kd), was shown to have a detectable carbohydrate component when the PI-PLC released sperm surface proteins were electrophoretically separated on SDS-PAGE and then stained for glycoproteins (Figure 1C). A Western blot of the same gel was probed with anti-PH-20 IgG, which revealed a band corresponding to the 64-kd protein stained for carbohydrates (Figure 1D). Further information about the oligosaccharides that are present on PH-20 was obtained by probing immunoaffinity-purified PH-20 with lectins that have known carbohydrate specificity. Of

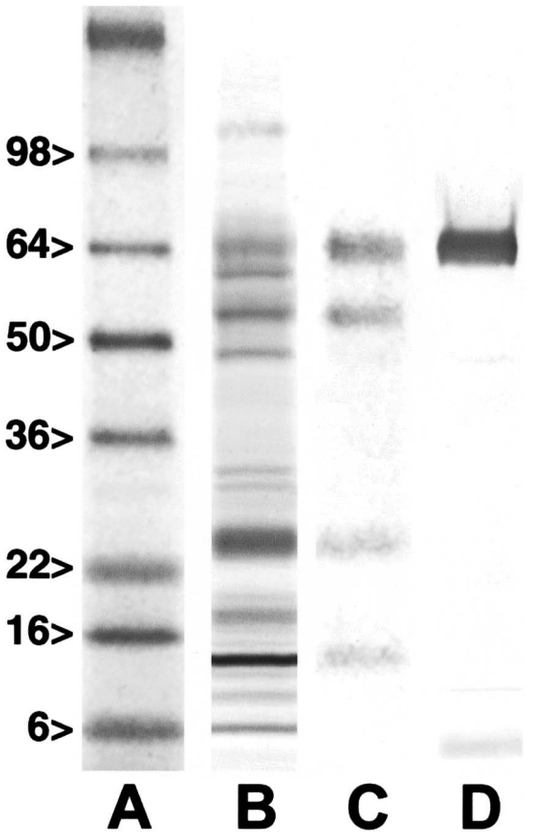

Figure 1. Cell surface proteins released after PI-PLC treatment of macaque sperm. Molecular weight markers are shown in lane A. The SDS-PAGE gel is stained silver to show the various proteins released (lane B). The gel is stained with Gel Code Glycoprotein Blue to identify glycoproteins (lane C) and with anti-PH-20 IgG to identify PH-20 (lane D).

the 22 different lectins tested, PSA, Con A, LEL, STL, PHA-E, and Jacalin recognized the 64-kd band (Figure 2). The other lectins tested did not recognize PH-20, or they recognized it faintly (not shown).

Immunoaffinity-purified PH-20 at low concentrations often appeared on the silver-stained gel as multiple bands with molecular weights of approximately 64 kd (Figure 3A), and when probed with anti-Peptide 1 IgG, an antibody raised to a peptide region of PH-20, the bands were easily discerned on a Western blot (Figure 3B). Analysis by 2-D PAGE revealed 6 isoforms of sperm surface PH-20, and their isoelectric points ranged from 5.1 to 6.0 (Figure 3C).

Oligosaccharides are joined to the polypeptide backbone at specific locations by either N- or O-linked bonds. N-glycosidase F hydrolyzes the N-linked bonds at the N-acetylglucosamine attachment to specific asparagine residues along the polypeptide. After deglycosylation of sperm surface PH-20 with N-glycosidase F, Western blot analysis of the digested protein with anti-PH-20 IgG revealed a major band with an apparent molecular weight of 54 kd (Figure 4B). Deglycosylation of PH-20 also produced a number of bands between 54 kd and 64 kd as a result of incomplete digestion (Figure 4D). Sperm surface PH-20 has hyaluronidase activity at pH 7.0 (Li et al, 1997), as shown in these experiments by the hyaluronic acid substrate gel assay (Figure 4C and E). Neither the 54-kd band nor the other bands of partially deglycosylated PH-20 had detectable hyaluronidase activity at pH 7.0 (Figure 4D and F). In contrast, the 64-kd band remaining after deglycosylation retained enzyme activity (Figure 4D and F). PH-20 was also subjected to enzyme removal of O-linked glycans, but there was no shift in the molecular weight or loss of hyaluronidase activity (data not shown).

When sperm surface PH-20 was treated with reducing reagent (β-mercaptoethanol or DTT) to break disulfide bonds, there was a shift in molecular weight from 64 kd to 67 kd, as shown previously (Li et al, 1997). After reduction, there was a complete loss of enzyme activity as assessed with the hyaluronic acid substrate gel assay (Figure 5B and C).

Discussion

The mammalian oocyte is surrounded by two extracellular matrices that the sperm must traverse prior to fertilization. Initially, the sperm passes through the cumulus oophorus, which is composed of granulosa cells embedded in a thick matrix of polymerized hyaluronic acid (Eppig, 1991). The cumulus matrix extends into the outer, porous region of the zona pellucida, a glycoprotein investment that the sperm also must penetrate (Tesarik and Kopecny, 1986; Katz et al, 1989; Wassarman, 1991). PH-20 has been shown to be involved in sperm passage through both the cumulus and the zona (Primakoff et al, 1985; Lin et al, 1994; Hunnicutt et al, 1996; Meyers et al, 1997; Yudin

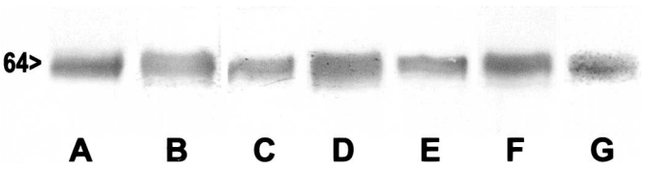

Figure 2. Lectin blot analysis of immunopurified sperm surface PH-20. The Western blot shows that anti-PH-20 IgG recognizes a single band of molecular weight 64 kd (lane A). The lectins Con A, PSA, LEL, STL, Jacalin, and PHA-E recognize this band, as shown in lanes B–G, respectively.

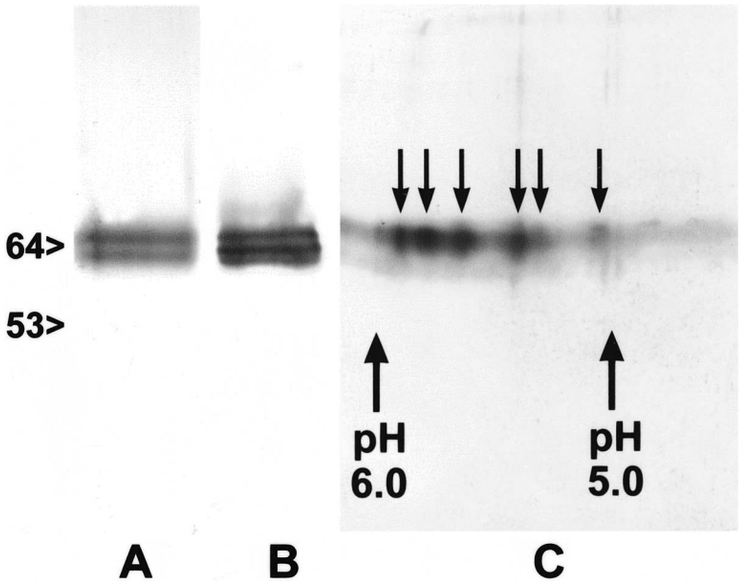

Figure 3. Western blot analysis of 1-D and 2-D SDS-PAGE of immunopurified sperm surface PH-20. The gel is stained with silver in lane A to reveal the protein bands. The multiple PH-20 bands are recognized by anti-Peptide 1 IgG (lane B). Panel **(C)** shows the Western blot analysis of 2-dimensional SDS-PAGE with anti-PH-20 IgG revealing 6 isoforms with isoelectric points ranging from 5.1 to 6.0.

et al, 1999). The various functions that have been attributed to the PH-20 protein include hyaluronidase activity, intracellular signaling, and zona pellucida adhesion and penetration (Myles et al, 1987b; Lin et al, 1994; Cherr et al, 1999; Yudin et al, 1999). The diverse functions of PH-20 were demonstrated initially by Hunnicutt et al (1996), who found that monoclonal antibodies that inhibit acrosome-reacted guinea pig sperm from binding to the zona pellucida did not block hyaluronidase activity, whereas the monoclonal antibody that inhibited hyaluronidase activity had no effect on sperm-zona binding. It stands to reason that the multiple functions attributed to PH-20 may result from variations in its secondary or tertiary structure, and the experiments described in this paper are a

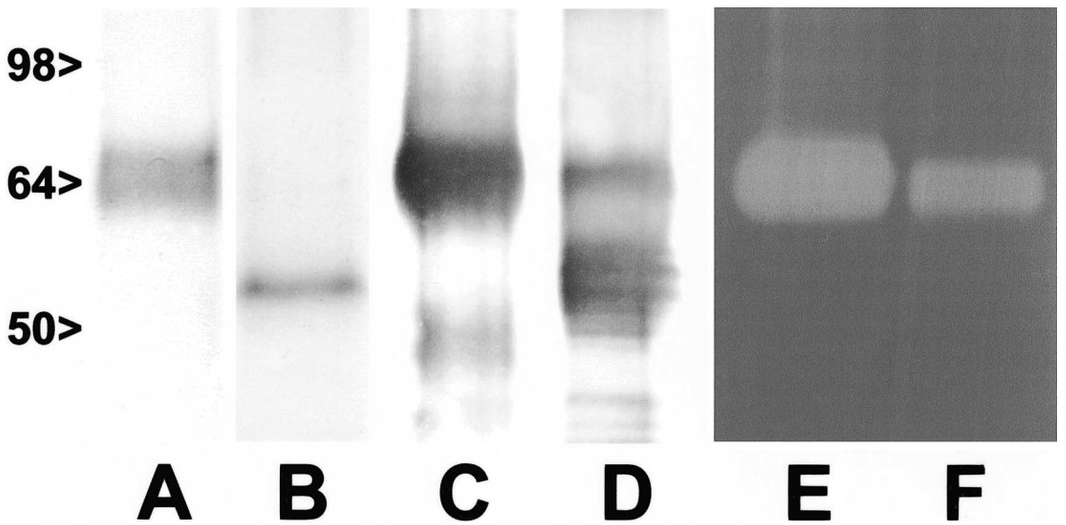

Figure 4. Western blot analysis and hyaluronic acid substrate gel analysis of immunopurified sperm surface PH-20 after treatment with N-glycosidase F. A single band at 64 kd is recognized by anti-PH-20 IgG in the control preparation (lane A). In the preparation treated with N-glycosidase F, a single band is recognized at 54 kd, but a band of intact PH-20 is still apparent at 64 kd (lane B). Minor bands representing PH-20 proteins with varying degrees of deglycosylation appear between 54 kd and 64 kd (lane C). The hyaluronic acid substrate gel analysis of this sample demonstrates hyaluronidase activity of the control preparation of PH-20 at pH 7.0 (lane E). The deglycosylated forms of PH-20, including the 54-kd band and the other minor bands, show no evidence of hyaluronidase activity, except that the 64-kd protein that remains following treatment with N-glycosidase F has hyaluronidase activity (lane F).

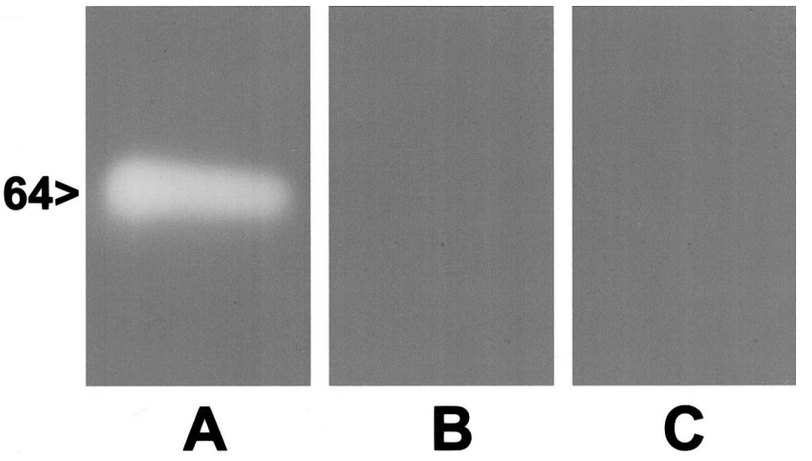

Figure 5. Hyaluronic acid substrate gel analysis of immunopurified sperm surface PH-20 after treatment with β-mercaptoethanol. Purified PH-20 (64 kd) prior to chemical reduction demonstrates hyaluronidase activity at pH 7 (lane A). After reduction with either β-mercaptoethanol or DTT there was no evidence of hyaluronidase activity (lanes B and C).

first attempt to gain information about these aspects of the PH-20 protein.

Amino acid sequence data have been published for a number of different hyaluronidases (Lathrop et al, 1990; Gmachl and Kreil, 1993; Lin et al, 1993; Lu et al, 1995). All of the hyaluronidases have numerous potential N-glycosylation sites characterized by the amino acid sequence Asp-X_{AA}-Ser/Thr, in which the internal X is not a proline (Struck and Lennarz, 1980; Osawa and Tsuji, 1987), although this sequence is not always glycosylated (Ronin et al, 1981). The addition of carbohydrate moieties is a common secondary gene product critical for biological or functional diversity (Kobata, 1992). Guinea pig sperm PH-20 has 6 potential glycosylation sites, and after treatment with N-glyanase, the molecular weight shifts approximately 10 kd; therefore, it is believed that most, if not all, of the sites are linked to oligosaccharides (Lathrop et al, 1990). This assumption is due to the similarity in molecular weight following deglycosylation (54 kd) and the theoretical molecular weight determined by amino acids (52 kd; Lathrop et al, 1990). Bovine PH-20 has 6 potential glycosylation sites, 2 of which are in the N-terminal region, but only one within the N-terminus appears to be glycosylated (Meyer et al, 1997). Macaque sperm PH-20 has at least 8 potential sites capable of N-glycosylation, and the molecular weight of sperm surface PH-20 falls approximately 20% after deglycosylation, suggesting multiple sites of glycosylation.

We propose that sperm surface PH-20 is glycosylated throughout the N- and C-terminal regions of the protein. This interpretation is consistent with the high degree of glycosylation (~20%) of the molecule. It is also based on the observation that when endoproteolyzed inner acrosomal membrane PH-20 is deglycosylated and reduced, there is a shift in the molecular weight of both the N- and C-terminal portions of the molecule, implying glycosylation within each region (unpublished data). Similar data are not available for sperm surface PH-20 because it is not endoproteolyzed (Li et al, 1997). Bee venom hyaluronidase is believed to be glycosylated at only 1 of 4 potential sites (Markovic-Housley et al, 2000). The addition of oligosaccharides is often cell specific, tissue specific, species specific, or a combination of these (Lis and Sharon, 1993); therefore, hyaluronidases may be glycosylated at similar sites, but the overall carbohydrate content could still be highly variable.

In Western blot analyses of macaque sperm surface PH-20 under nonreducing conditions, anti-PH-20 antibodies typically recognize a single broad band with an apparent molecular weight of 64 kd. Some antibodies, especially those built to short segments of PH-20, such as Peptide 1 (Yudin et al, 2001), will often recognize multiple bands within the broad 64-kd region, which is consistent with the results of 2-D PAGE showing 6 isoforms with isoelectric points ranging from 5 to 6. Initially, Primakoff et al (1988a) showed a single isoform for guinea pig PH-20 at pH 7.0. It was later reported that human PH-20 has a number of isoforms (Naaby-Hansen et al, 1997), but the molecular weights of the putative isoforms are different from the molecular weight proposed for human PH-20 (Naaby-Hansen et al, 1997; Sabeur et al, 1997). The various PH-20 isoforms are believed to be a reflection of the differences in glycosylation, which could arise at several stages in the processes of sperm production and maturation. PH-20 is derived from both the testis and the epididymis, and the epididymis also modifies sperm surface PH-20 during sperm maturation (Yanagimachi, 1994, for review; Phelps et al, 1999; Deng et al, 2000).

The present data show that removal of oligosaccharides from PH-20 eliminates its hyaluronidase activity. The re-

quirement of glycosylation for enzyme activity may not be a general feature of hyaluronidases. Gmachl and Kreil (1993) reported that a bacterial recombinant bee venom hyaluronidase had at least 80% of the activity of the native form, which suggests that glycosylation may not be critical for activity of the insect enzyme. Perhaps the single most important function of glycosylation is to aid in the folding of the molecule and stabilization of the conformation necessary for function (Lis and Sharon, 1993; Varki, 1993; Parodi, 2000). Glycosylation has been shown to be critical for the catalytic activity of a number of enzymes (Cumming, 1991; Howard et al, 1991). A single amino acid shift in the protein structure of saposin B results in loss of glycosylation at that site and, as a consequence, the rapid proteolysis and removal of the enzyme (Kretz et al, 1990). Hormones and receptors also have been shown to rely on glycosylation for affinity, with modification of the carbohydrate component of the hormone inducing a pronounced decrease in receptor binding (Ji and Ji, 1990; Zhang et al, 1991).

The glycan variability within the protein components of tissues and cells underlies much of the diversity observed in cell functions. One approach to investigation of this variability is to probe glycoproteins with specific lectins that have known carbohydrate specificity (Sumar et al, 1990). In most cases, lectins bind more strongly to oligosaccharides than monosaccharides, and the terminal sugar is most likely to be recognized (Goldstein and Poretz, 1986; Gravel and Golaz, 1996). The results of the present study suggest that mannose occurs as the terminal sugar or as a major component of the core oligosaccharide structure in sperm surface PH-20, whereas fucose and sialic acid appear not to be present. Jacalin has been proposed to recognize those oligosaccharides that are O-linked, but in our results, PH-20 appears to lack glycans that are O-linked. The existence of N-acetylglucosamines within the glycans was shown by labeling with both STL and LEL. Both lectins preferentially bind to trimers and tetramers of the sugar. PHA-E is a lectin with powerful red cell agglutination properties, and it recognizes complex carbohydrates.

A major structural feature of all hyaluronidases is the highly conserved cysteine residues, which are responsible for disulfide bonding (Lathrop et al, 1990; Gmachl and Kreil, 1993; Lin et al, 1993; Csoka et al, 1999). There are 3 cysteines in the N-terminal region of PH-20, one of these is linked by disulfide bonds to a cysteine in the carboxyl region, and this linkage holds the PH-20 molecule together after endoproteolytic cleavage (Lathrop et al, 1990). In bee venom hyaluronidase the cysteines 22 and 313 are linked, and 189 and 210 are also linked, thus forming an internal loop. The clustering of cysteines in the carboxyl region of PH-20 was proposed to contribute to the molecular structure required for close relationship of the molecule to the membrane (Lathrop et al, 1990). The present data demonstrate that removal of these loops within PH-20 by reduction of disulfide bonds with β-mercaptoethanol or DTT results in loss of hyaluronidase activity, probably because of changes in the conformation of the molecule. It is interesting to note that recombinant (bacterial) bee venom hyaluronidase retains its enzyme activity, although it is doubtful that any cysteine linkages are present. Two locations in the N-terminal region of PH-20 with highly conserved sequences of amino acids have been shown to be the active sites for hyaluronidase enzyme activity (Arming et al, 1997; Yudin et al, 2001), and a third site as been identified as the hyaluronic acid binding site responsible for intracellular signaling (Vines et al, unpublished observations). It is likely that the proximal relationships of these sites are altered after chemical reduction, and as a consequence, the enzyme activity of PH-20 is lost.

In conclusion, the PH-20 glycoprotein on the surface of cynomolgus macaque sperm is composed of 6 isoforms that differ in their carbohydrate composition. The hyaluronidase activity of the sperm surface PH-20 was lost when N-linked glycans were removed, and when sulfhydryl linkages in the protein were reduced. These results demonstrate that the enzyme activity of sperm surface PH-20 is dependent on the secondary and tertiary structure of the molecule.

References

Apweiler R, Hermjakob H, Sharon N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta.* 1999;1473:4–8.

Arming S, Strobl B, Wechselberger C, Kreil G. In vitro mutagenesis of PH-20 hyaluronidase from human sperm. *Eur J Biochem.* 1997;247: 810–814.

Bearer EL, Friend DS. Morphology of mammalian sperm membranes during differentiation, maturation, and capacitation. *J Electron Microsc Technol.* 1990;16:281–297.

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.

Cherr GN, Yudin AI, Li MW, Overstreet JW. Hyaluronic acid and the cumulus extracellular matrix induce increases in intracellular calcium in macaque sperm via the plasma membrane protein PH-20. *Zygote.* 1999;7:211–222.

Csoka AB, Scherer SW, Stern R. Expression analysis of six paralogous human hyaluronidase genes clustered on chromosomes 3p21 and 7q31. *Genomics.* 1999;60:356–361.

Cumming DA. Glycosylation of recombinant protein therapeutics: control and functional implications [see comments]. *Glycobiology.* 1991;1: 115–130.

Deng X, He Y, Martin-Deleon PA. Mouse Spam1 (PH-20): evidence for its expression in the epididymis and for a new category of spermatogenic-expressed genes. *J Androl.* 2000;21:822–832.

Eppig JJ. Mammalian oocyte development in vivo and in vitro. In: Wassarman PM, ed. *Elements of Mammalian Fertilization.* Boca Raton, Fla: CRC Press; 1991:57–76.

Gagneux P, Varki A. Evolutionary considerations in relating oligosaccharide diversity to biological function. *Glycobiology.* 1999;9:747–755.

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.

Goldstein IJ, Poretz RD. Isolation, physiochemical characterization, and carbohydrate-binding specificity of lectins. In: Leiner IE, Sharon N, Goldstein IJ, eds. *The Lectins: Properties, Functions, and Applications in Biology and Medicine.* Orlando, Fla: Academic Press; 1986: 35–247.

Gravel P, Golaz O. Identification of glycoproteins on nitrocellulose membranes using lectin blotting. In: Walker JM, ed. *The Protein Protocols Handbook.* Towota, NJ: Humana Press; 1996:603–613.

Howard SC, Wittwer AJ, Welply JK. Oligosaccharides at each glycosylation site make structure-dependent contributions to biological properties of human tissue plasminogen activator. *Glycobiology.* 1991;1: 411–418.

Hunnicutt GR, Primakoff P, Myles DG. Sperm surface protein PH-20 is bifunctional: one activity is a hyaluronidase and a second, distinct activity is required in secondary sperm-zona binding. *Biol Reprod.* 1996;55:80–86.

Ji I, Ji TH. Differential interactions of human choriogonadotropin and its antagonistic aglycosylated analog with their receptor. *Proc Natl Acad Sci USA.* 1990;87:4396–4400.

Katz DF, Drobnis EZ, Overstreet JW. Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments. *Gamete Res.* 1989;22:443–469.

Kobata A. Structures and functions of the sugar chains of glycoproteins. *Eur J Biochem.* 1992;209:483–501.

Kretz KA, Carson GS, Morimoto S, Kishimoto Y, Fluharty AL, O'Brien JS. Characterization of a mutation in a family with saposin B deficiency: a glycosylation site defect. *Proc Natl Acad Sci USA.* 1990; 87:2541–2544.

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.

Li MW, Cherr GN, Yudin AI, Overstreet JW. Biochemical characterization of the PH-20 protein on the plasma membrane and inner acrosomal membrane of cynomolgus macaque spermatozoa. *Mol Reprod Dev.* 1997;48:356–366.

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

Lis H, Sharon N. Protein glycosylation. Structural and functional aspects. *Eur J Biochem.* 1993;218:1–27.

Lu G, Kochoumian L, King TP. Sequence identity and antigenic cross-reactivity of white face hornet venom allergen, also a hyaluronidase, with other proteins. *J Biol Chem.* 1995;270:4457–4465.

Markovic-Housley Z, Miglierini G, Soldatova L, Rizkallah PJ, Muller U, Schirmer T. Crystal structure of hyaluronidase, a major allergen of bee venom. *Structure Fold Des.* 2000;8:1025–1035.

Meyer MF, Kreil G, Aschauer H. The soluble hyaluronidase from bull testes is a fragment of the membrane-bound PH-20 enzyme. *FEBS Lett.* 1997;413:385–388.

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.* 1997;46: 392–400.

Myles DG, Hyatt H, Primakoff P. Binding of both acrosome-intact and acrosome-reacted guinea pig sperm to the zona pellucida during in vitro fertilization. *Dev Biol.* 1987a;121:559–567.

Myles DG, Koppel DE, Cowan AE, Phelps BM, Primakoff P. Rearrangement of sperm surface antigens prior to fertilization. *Ann N Y Acad Sci.* 1987b;513:262–273.

Myles DG, Primakoff P. Localized surface antigens of guinea pig sperm migrate to new regions prior to fertilization. *J Cell Biol.* 1984;99: 1634–1641.

Naaby-Hansen S, Flickinger CJ, Herr JC. Two-dimensional gel electrophoretic analysis of vectorially labeled surface proteins of human spermatozoa. *Biol Reprod.* 1997;56:771–787.

Osawa T, Tsuji T. Fractionation and structural assessment of oligosaccharides and glycopeptides by use of immobilized lectins. *Annu Rev Biochem.* 1987;56:21–42.

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.

Parodi AJ. Role of N-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation. *Biochem J.* 2000; 348(part 1):1–13.

Phelps BM, Koppel DE, Primakoff P, Myles DG. Evidence that proteolysis of the surface is an initial step in the mechanism of formation of sperm cell surface domains. *J Cell Biol.* 1990;111:1839–1847.

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 cleaves PH-20 into two disulfide-linked fragments. *Biol Reprod.* 1988a;38:921–934.

Primakoff P, Hyatt H, Myles DG. A role for the migrating sperm surface antigen PH-20 in guinea pig sperm binding to the egg zona pellucida. *J Cell Biol.* 1985;101:2239–2244.

Primakoff P, Lathrop W, Woolman L, Cowan A, Myles D. Fully effective contraception in male and female guinea pigs immunized with the sperm protein PH-20. *Nature.* 1988b;335:543–546.

Ronin C, Granier C, Caseti C, Bouchilloux S, Van Rietschoten J. Synthetic substrates for thyroid oligosaccharide transferase. Effects of peptide chain length and modifications in the Asn-Xaa-Thr-region. *Eur J Biochem.* 1981;118:159–164.

Sabeur K, Cherr GN, Yudin AI, Primakoff P, Li MW, Overstreet JW. The PH-20 protein in human spermatozoa. *J Androl.* 1997;18:151–158.

Sarason RL, VandeVoort CA, Mader DR, Overstreet JW. The use of nonmetal electrodes in electroejaculation of restrained but unanesthetized macaques. *J Med Primatol.* 1991;20:122–125.

Struck DK, Lennarz WJ. The function of saccharide-lipids in synthesis of glycoproteins. In: Lennarz WJ, ed. *The Biochemistry of Glycoproteins and Proteoglycans.* New York, NY: Plenum Press; 1980:35–84.

Sumar N, Bodman KB, Rademacher TW, et al. Analysis of glycosylation changes in IgG using lectins. *J Immunol Methods.* 1990;131:127–136.

Tesarik J, Kopecny V. Late preovulatory synthesis of proteoglycans by the human oocyte and cumulus cells and their secretion into the oocyte-cumulus-complex extracellular matrices. *Histochemistry.* 1986; 85:523–528.

Varki A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology.* 1993;3:97–130.

Varki A, Freeze HH. The major glycosylation pathways of mammalian membranes. A summary. *Subcell Biochem.* 1994;22:71–100.

Wassarman PM. Construction of the zona pellucida during production of fertilizable mouse eggs. *Bull Assoc Anat (Nancy).* 1991;75:115–117.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction.* New York, NY: Raven Press; 1994: 189–317.

Yudin AI, Li MW, Robertson KR, Cherr GN, Overstreet JW. Characterization of the active site of monkey sperm hyaluronidase. *Reproduction.* 2001;121:735–743.

Yudin AI, VandeVoort CA, Li MW, Overstreet JW. PH-20 but not acrosin is involved in sperm penetration of the macaque zona pellucida. *Mol Reprod Dev.* 1999;53:350–362.

Zhang R, Cai H, Fatima N, Buczko E, Dufau ML. Functional glycosylation sites of the rat luteinizing hormone receptor required for ligand binding. *J Biol Chem.* 1995;270:21722–21728.

Zhang R, Tsai-Morris CH, Kitamura M, Buczko E, Dufau ML. Changes in binding activity of luteinizing hormone receptors by site directed mutagenesis of potential glycosylation sites. *Biochem Biophys Res Commun.* 1991;181:804–808.