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Mini review

The dual functions of GPI-anchored PH-20: hyaluronidase and intracellular signaling

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Abstract

The ovulated mammalian oocyte is surrounded by the 'cumulus ECM', composed of cells embedded in an extracellular matrix that is rich in hyaluronic acid (HA). The cumulus ECM is a viscoelastic gel that sperm must traverse prior to fertilization. Mammalian sperm have a GPI-anchored hyaluronidase which is known as PH-20 and also as SPAM 1. PH-20 is located on the sperm surface, and in the lysosome-derived acrosome, where it is bound to the inner acrosomal membrane. PH-20 appears to be a multifunctional protein; it is a hyaluronidase, a receptor for HA-induced cell signaling, and a receptor for the zona pellucida surrounding the oocyte. The zona pellucida recognition function of PH-20 was discovered first. This function is ascribed to the inner acrosomal membrane PH-20, which appears to differ biochemically from the PH-20 on the sperm surface. Later, when bee venom hyaluronidase was cloned, a marked cDNA sequence homology with PH-20 was recognized, and it is now apparent that PH-20 is the hyaluronidase of mammalian sperm. PH-20 is unique among the hyaluronidases in that it has enzyme activity at both acid and neutral pH, and these activities appear to involve two different domains in the protein. The neutral enzyme activity of plasma membrane PH-20 is responsible for local degradation of the cumulus ECM during sperm penetration. Plasma membrane PH-20 mediates HA-induced sperm signaling via a HA binding domain that is separate from the hyaluronidase domains. This signaling is associated with an increase in intracellular calcium and as a consequence, the responsiveness of sperm to induction of the acrosome reaction by the zona pellucida is increased. There is extensive evidence that GPI-anchored proteins are involved in signal transduction initiated by a diverse group of cell surface receptors. GPI-anchored proteins involved in signaling are often associated with signaling proteins bound to the cytoplasmic leaflet of the plasma membrane, typically Src family, non-receptor protein tyrosine kinases. PH-20 appears to initiate intracellular signaling by aggregating in the plasma membrane, and a 92-kDa protein may be the cell signaling molecule linked to PH-20. © 2001 Elsevier Science B.V./International Society of Matrix Biology. All rights reserved.

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1. Introduction

Mammals have two different extracellular matrices (ECMs) that surround the oocyte (Yanagimachi, 1994, for review). The sperm initially traverses a viscoelastic hyaluronic acid (HA)-protein ECM, the 'cumulus ECM', which is fully expanded at ovulation (Eppig, 1991; Salustri et al., 1999). The cumulus ECM extends into the outer porous region of the glycoprotein ECM, the zona pellucida, that sperm must penetrate before fertilizing the egg (Yanagimachi, 1994, review). The cumulus ECM is dependent on protein–HA interactions for its elasticity, as shown by protease treatment, which results in a highly viscous gel with no elasticity (Cherr et al., 1990). The cumulus ECM can be degraded by a variety of hyaluronidases (Cherr et al., 1990).

Mammalian testes have long been recognized as a rich source of what was referred to as 'spreading factor' and was later found to be hyaluronidase (Chain

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and Duthie, 1939). Some years later, sperm-derived hyaluronidase was identified as being involved in mammalian gamete interaction (Austin, 1960). While the existence of a sperm derived hyaluronidase became widely accepted, its function during fertilization remained controversial. Soluble hyaluronidase was known to be released from sperm at the time of the acrosome reaction, but Saling and Storey (1979) demonstrated that sperm pass through the cumulus in an acrosome-intact state and acrosome react after reaching the zona. A hyaluronidase on the sperm surface was eventually shown to hydrolyze HA, and was implicated in penetration of the cumulus (Lewin et al., 1982). When bee venom hyaluronidase was cloned, a marked cDNA sequence homology with the N-terminal region of PH-20 was recognized (Gmachl and Kreil, 1993; Lin et al., 1993), and it became apparent that PH-20 was likely to have hyaluronidase activity. This prediction was confirmed in experiments with purified PH-20 (Gmachl and Kreil, 1993; Lin et al., 1994; Cherr et al., 1996).

There is now strong evidence that PH-20 is the only hyaluronidase in mammalian sperm, including sperm of guinea pigs (Hunnicutt et al., 1996a), rats (Hou et al., 1996), macaques (Cherr et al., 1996; Li et al., 1997a) and humans (Sabeur et al., 1997). The well-known 'bovine testicular hyaluronidase' is also PH-20 (Meyer et al., 1997). Prior to these discoveries, almost a decade of research had characterized PH-20 as a sperm receptor for zona pellucida (Primakoff et al., 1985; Myles et al., 1987; Hunnicutt et al., 1996b). Now there is evidence that PH-20 is essential for sperm penetration of both the cumulus ECM and the zona pellucida (Primakoff et al., 1985; Lin et al., 1994; Hunnicutt et al., 1996b; Meyers et al., 1997; Li et al., 1997b; Yudin et al., 1999). Recently it was shown that mice bearing the Robertsonian translocation (Rb 6.16 or 6.15) results in sperm with both a qualitative and quantitative deficiency in PH-20 hyaluronidase activity (Zheng et al., 2001). This genetic dysfunction results in a clear reduction in fertility based on litter size (Zheng et al., 2001).

A number of ubiquitous and tissue-specific forms of mammalian hyaluronidase have been identified including HYAL 1, 2, 3 and 4 (Csoka et al., 1999). Although the mammalian hyaluronidases are derived from different genes, these enzymes share conserved structural and biological characteristics with one another and with other glycosidases. PH-20 has varying degrees of sequence homology with each of these hyaluronidases, but it appears to be the only hyaluronidase with significant enzyme activity at neutral pH. Recently HYAL 2 was shown not only to be found in lysosomes, but also, like PH-20, to be expressed on the cell surface as a GPI-anchored protein (Rai et al., 2001).

Although there are conflicting data, Northern and Southern blots of various tissues suggest that there is a single testis-specific gene for PH-20 (Li et al., 1993; Jones et al., 1995). Sperm leaving the testes have GPI-anchored PH-20 over their entire surface, as well as, on the sequestered inner acrosomal membrane (Phelps and Myles, 1987; Phelps et al., 1990; Deng et al., 1999). Although Northern blots of the epididymal tissue were negative for PH-20, there is strong evidence that PH-20 is actively secreted by the principal cells in the epididymal lumen (Deng et al., 1999; Zhang and Martin-DeLeon, 2001). During sperm transit through the epididymis, additional PH-20 is assumed to be adsorbed to the sperm surface (Deng et al., 2000). This phenomenon could reflect a redundant expression of PH-20, or it could represent a mechanism for insertion of an isoform of PH-20 that is different from those expressed in testes (Zhang and Martin-DeLeon, 2001). By the time sperm reach the cauda region of the epididymis, PH-20 is proposed to undergo a reduction in molecular weight, as well as, relocation and restriction to the sperm head membrane (Phelps and Myles, 1987). Inexplicably, the 2–3 kDa reduction in molecular weight is also observed for PH-20 on the inner acrosomal membrane, which is not exposed to epididymal glycosidases or proteases.

Cell surface hyaluronidase activity, as well as flagellar activity, is important for sperm penetration of the HA-rich cumulus ECM (Cherr et al., 1990). Because the HA in cumulus ECM is organized in a macromolecular gel, it can efficiently crosslink multiple sites on the sperm plasma membrane that bind HA. We now recognize that such HA binding to the sperm membrane and subsequent intracellular signaling events in sperm are mediated by PH-20 (Cherr et al., 1999; Vines et al., 2001). It is accepted that most mammalian sperm do not undergo the acrosome reaction until they contact the zona pellucida, where the zona glycoproteins initiate this exocytotic event (Wassarman, 1990, for review). Only at this time is the soluble form of PH-20 released from the sperm cell, and the function of this hyaluronidase remains unclear, since like the HYAL proteins, it is active only at acid pH (Cherr et al., 1996). Following the acrosome reaction, the bulk of PH-20 remains on the inner acrosomal membrane. There is evidence that PH-20 on the inner acrosomal membrane participates in secondary or tertiary sperm-zona binding as well as in sperm penetration of the zona pellucida (Hunnicutt et al., 1996b; Yudin et al., 1999), but the details of these interactions are also unclear. In this review we will focus on the hyaluronidase and cell signaling functions of PH-20 that operate during sperm penetration of the cumulus.

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2. The hyaluronidase activity of PH-20

The glycosidases characteristically have two acidic amino acids that function as either the acid/base catalyst site or the nucleophile site (Braithwaite et al., 1997; Zverlov et al., 1998). Initially, Arming et al. (1997) constructed a variety of recombinant human PH-20 proteins that were mutated at sites with highly conserved acidic and basic amino acids. All of the mutated proteins were shown to be deficient in hyaluronidase activity at neutral pH. We investigated the active site of macaque PH-20 using affinity purified Fab fragments of antibodies constructed against selected peptide regions (Yudin et al., 2001). Two potential catalytic sites for PH-20 were identified. The region designated Peptide 1 encompasses the amino acid sequence 142–172 (AVIDWEEWRPTWARNWKPKDVYKNRSIELV). This region is approximately 86% conserved from human to mouse PH-20, but when compared to the HYAL proteins, human PH-20 ranges from as low as 46% conserved for HYAL 3–66% for HYAL 1. A second region with characteristics of an enzyme catalytic site was designated Peptide 3 and includes the amino acid sequence 277–297 (YVRNRVREAIRVSKIPDAKN). This region of PH-20 is approximately 70% conserved from human to mouse, but has less than 40% homology when HYAL proteins are compared with PH-20. Plasma membrane PH-20 has hyaluronidase activity only at neutral pH, while inner acrosomal membrane PH-20 has activity at both neutral and acid pH (Li et al., 1997a; Yudin et al., 2001). Our data suggest that the Peptide 1 region is essential for enzyme activity of PH-20 at neutral pH, and it is likely to be the acid/base region of the enzyme. The Peptide 3 region appears to be important at a lower pH and may be the nucleophilic site of PH-20 (Yudin et al., 2001).

The Peptide 1 region is characterized by an abundance of aromatic amino acids flanking the catalytic residue, glutamic acid, a common feature of other glycosyl hydrolases (Van Roey et al., 1994). Another common feature of these enzymes is the presence of basic amino acids or an organized region of basic amino acids which are utilized for substrate HA binding or docking (Withers and Aebersold, 1995), and this is also present in the Peptide 1 region. Further support for the Peptide 1 region being the primary active site of PH-20 comes from Chan et al. (1999) who found that antibodies to PH-20, which caused infertility in female guinea pigs, had highest titers to a region of the PH-20 molecule corresponding to the Peptide 1 region.

Markovic-Housley et al. (2000) have published the crystal structure of bee venom hyaluronidase and have shown that the overall topography includes a groove or cleft that extends perpendicular to the barrel axis. The groove is approximately 30 A × 10 A which is large enough to contain at least a hexasaccharide of HA. The 'valley' of the groove has a high concentration of aromatic residues that are conserved throughout all of the hyaluronidases, and this location is considered the active site. The Peptide 1 region of macaque PH-20 (Yudin et al., 2001) is located along one of the walls of the active site groove (Markovic-Housley et al., 2000).

The Peptide 3 site corresponds to one of the inactive mutations made by Arming et al. (1997) and to the site of a natural mutation at amino acid 268 of HYAL 1,which resulted in a lack of enzyme activity at acid pH in the affected individual (Triggs-Raine et al., 1999). In our study, we found that the antibody to Peptide 3 region did not inhibit hyaluronidase activity of plasma membrane PH-20 at neutral pH (pH 7.0); however, antibodies to both Peptide 3 and Peptide 1 were capable of inhibiting the acid-active (pH 5.0) activity of PH-20 obtained from the inner acrosomal membrane following the acrosome reaction (Yudin et al., 2001). These findings suggest that PH-20 attains the capability of acid-active hyaluronidase activity after the acrosome reaction, and that the Peptide 3 region is involved in acid-active enzyme activity, as appears to be the case for HYAL 1.

At the time of the acrosome reaction, PH-20 is endoproteolytically cleaved (between amino acids 312 and 313) but is held together after cleavage by a disulfide bond (Primakoff et al., 1988). This endoproteolysis may alter the three-dimensional structure of PH-20, enabling the Peptide 3 region to become active and thus imparting both neutral- and acid-active activities to PH-20. A major structural feature of all hyaluronidases is the highly conserved cysteine residues and their potential for disulfide bonding (Lathrop et al., 1990; Gmachl and Kreil, 1993; Li et al., 1993; Csoka et al., 1999). It is likely that the four highly conserved cysteines create the loops and stabilize the groove that has been shown in bee venom hyaluronidase (Markovic-Housley et al., 2000). In PH-20 there are two cysteines in the N-terminal region, one of which is linked to the cysteine at approximately 316aa, which is just past the endoproteolytic cleavage site; this linkage holds the molecule together following endoproteolysis (Lathrop et al., 1990). Reducing agents completely eliminate all hyaluronidase activity of PH-20 (Li et al., 2001). We propose that the removal of the loops formed by disulfide bonds in PH-20 eliminates hyaluronidase activity by radically modifying the conformation. Unfortunately, only a subset of PH-20 is endoproteolyzed or capable of being cleaved; therefore, it is difficult to identify the physiological changes that occur in PH-20 after proteolysis.

The functions of a multifunctional glycoprotein may

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be regulated by its secondary structure. N-Linked glycans play a major role in the regulation of protein conformation and ultimate stabilization of intramolecular folding that is critical for biological activity (Lis and Sharon, 1993; Varki, 1993; Zhang et al., 1995). Amino acid sequence data have been published for a number of different hyaluronidases (Gmachl and Kreil, 1993; Lu et al., 1995; Lin et al., 1993; Lathrop et al., 1990). All of the hyaluronidases have numerous potential N-glycosylation sites within the amino acid sequence Asp-X_{AA}-Ser/Thr, in which the internal X is not a proline (Osawa and Tsuiji, 1987; Struck and Lennarz, 1980). Guinea pig sperm PH-20 has six potential glycosylation sites, and after treatment with N-glycanase, the molecular weight shifts approximately 10 kDa. Therefore, it is believed that most, if not all, of these sites have oligosaccharides (Lathrop et al., 1990). Macaque sperm PH-20 has at least eight sites capable of N-glycosylation, and has $\sim 20\%$ reduction in apparent molecular mass following deglycosylation (Li et al., 2001), which is a similar response to that observed for guinea pig PH-20 (Lathrop et al., 1990). While hyaluronidases may have the common feature of glycosylation at similar sites, the specific oligosaccharides may be quite diverse. For example, mouse sperm PH-20 is expressed by the epididymis as well as the testis (Deng et al., 2000; Zhang and Martin-DeLeon, 2001), and it is also known that the epididymis can modify enzymatically plasma membrane glycoproteins (Yanagimachi, 1994, for review). Although it has not been investigated, we speculate that plasma membrane PH-20 may have subtle differences in glycosylation as compared to inner acrosomal membrane PH-20, due to the temporal and spatial sorting of PH-20 within the testes and the additional production and modification of PH-20 within the epididymis. Whether such glycosylation differences, the difference in endoproteolysis of the two forms of the protein, or both of these phenomena are responsible for differences in pH optima of enzyme activity remains to be determined.

The broad, bimodal pH range of PH-20 (from 4.5 to 8 in primate sperm; Cherr et al., 1996; Li et al., 1997b) is due, in part, to a 53-kDa form of PH-20 that appears after the acrosome reaction and is at least partially released from the inner acrosomal membrane (Cherr et al., 1996). This 53-kDa form of PH-20 is acid-active with an optimum at approximately pH 4.5 (Li et al., 1997a). This unique shift in pH activity may be the result of a proteolytic cleavage in the carboxyl region of the molecule or removal of glycosylation sites (Meyer et al., 1997). Bee venom hyaluronidase is acid-active and does not possess the carboxyl terminal 150 amino acids, but when recombinant PH-20 was produced without the carboxyl region there was a complete loss of activity (Gmachl and Kreil, 1993; Arming et al., 1997). The acid-active 53-kDa PH-20 only appears after the acrosome reaction and only accounts for approximately 10% of the PH-20, regardless of the percentage of reacted sperm or the duration of sperm incubation following the reaction. This observation suggests that only a subpopulation of PH-20 on the inner acrosomal membrane is susceptible to enzymatic modification, or that a very transient enzymatic reaction occurs (Cherr et al., 1996). It has been proposed that the acid-active soluble 53-kDa PH-20 may be functionally important in clearing a space in the cumulus ECM around the head of the sperm, thus facilitating repositioning of the sperm head for penetration into the zona (Drobnis et al., 1988; Cherr et al., 1996). In this model, the area of hydrolysis is limited by the return of the microenvironment to a physiological pH, thereby rendering the soluble 53-kDa PH-20 inactive.

The glycosylation state of the enzyme may be a key similarity between the acid-active 53-kDa PH-20 and the acid-active HYAL proteins. The secondary structures of the hyaluronidases including bee venom, PH-20 and the HYAL proteins, have highly conserved active sites as well as conserved cysteines (Lin et al., 1993; Gmachl and Kreil, 1993; Csoka et al., 1999). Bee venom hyaluronidase is acid-active and has three potential N-glycosylation sites, but only one site appears to be glycosylated (Markovic-Housley et al., 2000). HYAL 1, the serum hyaluronidase, has a very strict and low pH optimum of approximately 4 (Frost et al., 1997a; Csoka et al., 1997). HYAL 1 has a theoretical molecular weight of 49 kDa, but posttranslational glycosylation results in an observed molecular weight of approximately 57 kDa (Csoka et al., 1997). HYAL 2 has a similar acid-active profile (Strobl et al., 1998). HYAL 1, 2 and 3 have three potential glycosylation sites, while PH-20 has been shown to have seven or eight sites, depending on the species. This variation in glycosylation may modulate biological activity and confer different properties on different enzymes.

There are a number of reports of PH-20 in cancer cells (Liu et al., 1996; Madan et al., 1999), and it appears that hyaluronidase(s) are involved in tumor invasion and metastasis (West and Kumar, 1989; Lokeshwar et al., 1996; Tamakoshi et al., 1997; Csoka et al., 1997; Lokeshwar et al., 1998, 1999; Madan et al., 1999; Victor et al., 1999). Podyma et al. (1997) showed that human carcinoma cells expressed six biologically active isoforms of HYAL 1, while only two forms were expressed in normal tissue. They also found that by blocking the glycosylation process, a complete loss of hyaluronidase activity was induced (Podyma et al., 1997). We observed a similar loss of hyaluronidase activity when PH-20 was incubated with *n*-glycanase (Li et al., 2001). Neuramindase treatment of HYAL 1 from carcinoma cells resulted in a re-

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duced molecular weight and a marked increase in hyaluronidase activity (Podyma et al., 1997). Lokeshwar et al. (1999) reported that bladder cancer cell lines produce 10–100 times more of HYAL 1 than do normal bladder cells and that the molecular weight of this isoform is higher than that of the normal enzyme. Unlike the HYAL 1 of normal cells, which has no activity at pH 4.5, the isoform from malignant cells retains at least 80% of the activity at pH 4.5 (Lokeshwar et al., 1999). The HYAL 1 of invasive cancer cells may have a different pattern of glycosylation that modifies its hyaluronidase activity (Maingonnat et al., 1999). Macaque sperm surface PH-20 has at least six isoforms, all of which are active at neutral pH (Li et al., 2001). The lower pH optimum of the 53-kDa acid-active form of PH-20 may result from action of glycosidases (Cherr et al., 1996) rather than a post-translational modification as seen in HYAL proteins. The expression of PH-20 in cancer cells has been suggested and requires confirmation, but it seems likely that an up-regulation HYAL proteins does occur in cancer cells with a consequent increase in hyaluronidase activity that is associated with metastasis (Pham et al., 1997; Tamakoshi et al., 1997; Victor et al., 1999; Lokeshwar et al., 1999).

3. A role for PH-20 in hyaluronic acid-induced sperm signaling

Hyaluronic acid, the substrate for hyaluronidase, is now known to have important functions in angiogenesis, cell motility, wound healing, fertilization, embryogenesis, and cancer progression (Frost et al., 1997b; Delpech et al., 1997; Toole, 1997, 2000; Collis et al., 1998; Lokeshwar et al., 1999; Madan et al., 1999; Camenisch et al., 2000). Recently, there have been numerous investigations to identify the cell surface proteins that interact with HA and regulate its signal transduction activity (Aruffo, 1996; Bourguignon et al., 1997; Fitzgerald et al., 2000; Oliferenko et al., 2000; Stern et al., 2001). Cell surface receptors that may be involved in HA-signaling include CD44, RHAMM, and TSG-6 (Toole, 1990; Neame and Barry, 1993; Iozzo and Murdoch, 1996; Cheung et al., 1999; Knudson et al., 1999).

In both human and macaque sperm, HA induces an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) through an interaction with PH-20 (Sabeur et al., 1998; Cherr et al., 1999). It has been proposed that the binding of HA in the cumulus ECM to sperm surface PH-20 results in aggregation of the PH-20 protein, causing this signaling event (Cherr et al., 1999). The increase in $[Ca^{2+}]_i$ that occurs when sperm encounter HA is not sufficient to trigger the acrosome reaction, but may function synergistically with zona pellucida glycoproteins during induction of the acrosome reaction after sperm binding to the zona.

3.1. Identification of a PH-20 HA binding domain responsible for cell signaling

Gaseca et al. (1994) identified a potential HA binding domain that is within one of the catalytic sites of PH-20 and is similar to the HA binding domain described for RHAMM (Yang et al., 1993). We have recently investigated a different putative HA-binding domain of macaque sperm plasma membrane PH-20 (termed 'Peptide 2') and examined its function in HA-induced cell signaling (Vines et al., 2001). The Peptide 2 region (amino acids 205–235 with boxes representing putative HA-binding regions of the peptide: KLGRSLRPNHLWGYYLFPDCYNHHYRKPGYN) of the PH-20 molecule contains a cluster of basic amino acids with neutral amino acids in between (BxxBxxB), an arrangement that is similar to the HA binding motif of RHAMM (B(X7)B) (Yang et al., 1993). This region is highly conserved in hyaluronidases and is similar to the heparin binding motif (BxxBxBxxB) (Sobel et al., 1992). The presence of an HA binding motif in this region of PH-20 is supported by binding of HA to a recombinant protein (amino acids 143–510) which encompasses the Peptide 2 region, but not to the recombinant protein E12 (amino acids 291–510), which lacks the Peptide 2 region. We have also shown that the Peptide 2 domain mediates the HA-induced increase in sperm $[Ca^{2+}]_i$ by blocking this activity with an affinity purified Fab fragment of anti-Peptide 2 IgG. Arming et al. (1997) substituted the highly conserved terminal arginine within this motif and found that it caused a substantial loss of hyaluronidase activity, which would suggest that HA binding in this region also is necessary for hyaluronidase activity. However, in contrast to the inhibition of macaque sperm hyaluronidase activity by Fab fragments of IgG from anti-Peptides 1 and 3 (Yudin et al., 2001), Fab fragments of anti-Peptide 2 IgG did not inhibit macaque sperm hyaluronidase activity (Vines et al., 2001). Taken together, these results provide direct evidence that primate PH-20 has an HA binding domain that is distinct from the hyaluronidase domains (Yudin et al., 2001).

3.2. Possible mechanisms of PH-20 signal transduction

It has been established that HA is involved in signal transduction in a number of cell types, and some of these are analogous to signaling events we observe in sperm exposed to HA. HA is known to stimulate protein kinase activity in intact fibroblasts (Turley, 1989), it mediates motility via phosphoryla-

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tion of an HA binding protein in sperm flagella (Ranganathan et al., 1995) and it induces phosphorylation of an HA binding protein in lymphocytes (Rao et al., 1996). More recently, HA has been shown to be involved in promotion of tumor activation, angiogenesis, and inflammatory responses. These examples of HA signaling include: (1) ovarian carcinoma cells, where CD44 binds HA and then interacts with $p185^{HER2}$ to signal human ovarian carcinoma development (Bourguignon et al., 1997); (2) T lymphoma cells, where HA binding increases $[Ca^{2+}]_I$, interaction with ankyrin, and patching and capping of the HA receptor (Bourguignon et al., 1993); (3) chemokine gene expression in alveolar macrophges (McKee et al., 1996); and (4) nitric oxide synthase activation in macrophages (McKee et al., 1997).

Our findings that the GPI-anchored plasma membrane hyaluronidase in primate sperm is a receptor for HA and that it mediates intracellular calcium increases (Sabeur et al., 1998; Yudin et al., 1998; Cherr et al., 1999; Vines et al., 2001) at first appeared to be novel. However, recently the GPI-anchored HYAL 2 was found to be enzymatically inactive, and instead functions as a receptor for Jaagsiekte retrovirus, which induces transformation in fibroblasts leading to oncogenesis (Rai et al., 2001; see Lepperdinger et al., 2001 this volume). HYAL 2 is 35.2% identical to PH-20 and in the Peptide 2 region it is 60% identical (Lepperdinger et al., 1998, 2001). Whether the homologue of the PH-20 Peptide 2 region is involved in HYAL 2 retrovirus recognition is unknown, but functional comparisons between HYAL 2 and PH-20 would enable us to begin to understand the multifunctional roles of GPI-anchored hyaluronidases.

Our laboratory provided the first evidence that PH-20 interacts with HA to potentiate the zona pellucida-induced acrosome reaction (VandeVoort et al., 1997b; Sabeur et al., 1998), and we showed that the mechanism of this function involves $[Ca^{2+}]_I$ increases (Sabeur et al., 1998; Yudin et al., 1998; Cherr et al., 1999), possibly through clustering of PH-20. GPI-anchored proteins such as PH-20 are implicated in the signal transduction initiated by a diverse group of cell surface receptors, even though these GPI-anchored proteins do not have a cytoplasmic signal domain (Brown, 1993; Robinson, 1997; Horejsi et al., 1998; Ilangumaran et al., 1999). GPI-linked proteins are known to communicate with the intracellular environment, which was originally perplexing given that they are inserted into the outer leaflet of the plasma membrane (Robinson, 1991). The GPI-anchored proteins involved in signaling are always associated with signaling molecules that are typically bound to the cytoplasmic leaflet of the membrane. These proteins include the Src-family of tyrosine kinases. These kinases have been isolated by treating cells with antibodies to the GPI-anchored proteins followed by detergent extraction and immunopreciptation of the GPI-anchored protein complex (Harder et al., 1998; Horejsi et al., 1998). The GPI-anchored proteins and the Src-family tyrosine kinases appear to be located in glycosphingolipid-cholesterol microdomains (so-called 'lipid rafts'), since GPI-anchored proteins have saturated acyl chains and are likely to be preferentially inserted into these lipid domains (reviewed by Kasahara and Sania, 2000). It has been demonstrated that signaling can occur following aggregation and crosslinking of the GPI-anchored protein and that the patches that form around the aggregated proteins are enriched in tyrosine phosphorylated proteins as well as tyrosine kinase (Harder and Simon, 1997).

GPI-anchored proteins are often resistant to detergent extraction (Triton X-100) and are referred to as detergent insoluble glycolipids (DIG), which float on top of sucrose gradients (Ferguson et al., 1999). The DIG fraction is highly enriched in sphingolipids, cholesterol, GPI-anchored proteins, non-receptor tyrosine kinases and g-proteins (Kasahara and Sania, 2000). Currently there are at least 200 identified GPI-anchored proteins, all of which are highly glycosylated and have multiple isoforms (Ferguson, 1999; Fivaz et al., 2000). These DIG fractions are reported to form platforms of supra-molecular complexes that enable the concentration of receptors and signal transducers on both sides of the membrane (Simon and Ikonian, 1997; Brown and London, 1998). Kenworthy and Edinin (1998) reported that they could not detect these platforms using FRET-microscopy, but Varma and Mayor (1998) proposed that often the microdomains are below the resolution of microscopy. Harder et al. (1998) found that not all GPI-anchored proteins are within microdomains and those within the microdomains would co-aggregate with specific antibodies.

The GPI-anchored plasma membrane PH-20 on the surface of capacitated macaque sperm, when localized fine structurally, shows an even distribution along the head (Overstreet et al., 1995). PH-20 on the sperm surface is probably expressed and inserted by two different tissues and by two different cellular processes (Phelps et al., 1988; Deng et al., 2000; Zheng et al., 2001). It is not known if GPI-anchored protein expressed and secreted into the epidiymal lumen occurs via lipid vesicles that contain a complex of GPI-linked proteins and signaling molecules, or if they are individually secreted (Kirchhoff and Hale, 1996; Minelli et al., 1999; Deng et al., 2000; Zhang and Martin-DeLeon, 2001). PH-20 has been shown to move along the plasma membrane at the same lateral mobility as reported for integral membrane proteins, which would suggest that it exists in a complex, as

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seen in other cell types (Primakoff et al., 1988). This could explain why a substantial population of PH-20 molecules is found to be Triton X-100 extractable (Cherr et al., 1996). We speculate that PH-20 along the inner acrosomal membrane may not be in sphingolipid-cholesterol microdomains as it is on the plasma membrane, since there is probably little need for the inner acrosomal membrane to communicate with the internal cytoplasmic environment. Recently Ohta et al. (2000) found that the sea urchin sperm receptor for the egg ligand was found in the DIG fraction, which had at least four proteins involved in signal transduction, including a 63-kDa GPI-anchored protein. Specific antibodies to either the egg receptor or the GPI-anchored 63-kDa protein would co-immunoprecipitate this complex of receptors, the GPI-anchored protein, and other putative signal transducers (Ohta et al., 2000). Antibodies to specific GPI-anchored proteins have been shown to co-precipitate a variety of tyrosine kinases (src, fyn, lyn and csk), which are believed to regulate signal transduction (Harder et al., 1998; Prinetti et al., 1999).

We now have data suggesting the existence of a protein associated with PH-20 in the plasma membrane of macaque sperm that may be involved in signal transduction. To isolate this protein, we incubated live capacitated sperm with anti-PH-20 IgG. After washing through Ficoll to remove free IgG, sperm were extracted in Triton X-100/OG buffer containing protease inhibitors. The extract was then subjected to Protein A-agarose affinity chromatography to isolate IgG/PH-20/protein complexes. These complexes were eluted off of the Protein A-agarose after washing and were analyzed by SDS-PAGE with silver staining as well as immunoblotting. These complexes consist of plasma membrane PH-20 (64 kDa), the anti-PH-20 IgG (150 kDa), and a 92-kDa protein (Fig. 1b). A polyclonal antibody was raised against this 92-kDa protein and this anti-92-kDa IgG recognizes only the 92-kDa protein in whole sperm on Western blots (Fig. 1c). We have also observed that a 92-kDa protein is one of the proteins that undergoes tyrosine phosphorylation when sperm are treated with HA or anti-PH-20 IgG (Fig. 1d), but not with Fab fragments of anti-PH-20 IgG (not shown). The phosphorylation of tyrosine by HA is inhibited when sperm are pretreated with Fab fragments of anti-PH-20 IgG.

Work is now in progress to complete the characterization of the PH-20-associated 92-kDa protein and to determine if it has kinase activity and if it is the same protein that is tyrosine phosphorylated in sperm treated with HA or anti-PH-20 IgG. We are also determining if this protein is associated with the internal leaflet of the plasma membrane bilayer or if it is a transmembrane protein that has an external domain that interacts with PH-20 other than at the GPI anchor.

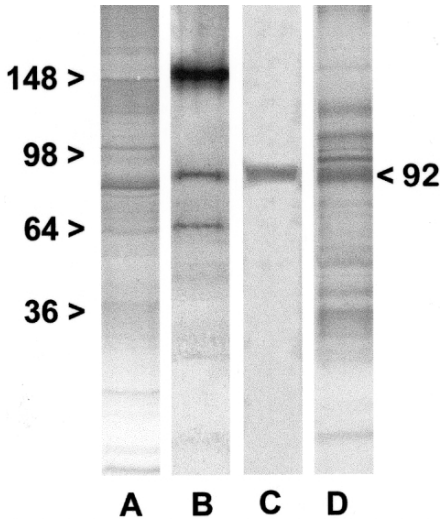


Fig. 1. (a) Silver stain SDS-PAGE of whole macaque sperm. (b) Isolate of PH-20 following aggregation with anti-PH-20 IgG. A ~92-kDa protein is co-isolated with PH-20 using the anti-PH-20 antibody. The band at 150 kDa is the anti-PH-20 IgG and the 64-kDa protein is PH-20. (c) A polyclonal antibody raised against the 92-kDa protein recognizes a single band in whole sperm that corresponds to the purified protein. (d) Whole sperm treated with HA and probed with an antibody to phosphotyrosine (RC20;Transduction Labs). There is a prominent band at the 90–92-kDa region. Identical results are obtained in sperm treated with anti-PH-20 IgG. All molecular weights shown are in kilodaltons (kDa).

4. Summary

PH-20 is a GPI-anchored hyaluronidase that has both enzymatic and signaling activity. This sperm protein is novel in that it is multi-functional: (a) the hyaluronidase activity (neutral-active on the cell surface, acid and neutral on the inner acrosomal membrane) is required for penetration of the cumulus ECM, and the active domains show some degree of homology with HYAL proteins; (b) the binding of HA by plasma membrane PH-20 via a distinct HA-binding domain results in tyrosine phosphorylation and increases in intracellular Ca^{2+}, possible via a 92-kDa associated protein; and (c) inner acrosomal membrane PH-20 is a zona-recognition molecule in some species, and at the very least, plays a key role in sperm interaction with and penetration of the zona pellucida. While the PH-20 protein was thought to be sperm-specific, there are indications that it could be expressed in metastatic cancer cells. The functional domains of such a PH-20 homologue are unknown. However, by studying the normal isoforms of sperm PH-20 and their different biological properties, we

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will gain new insights into the function of similar proteins in cancer cells.

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References

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

Aruffo, A., 1996. CD44: one ligand, two functions [editorial]. J. Clin. Invest. 98, 2191–2192.

Austin, C.R., 1960. Capacitation and the release of hyaluronidase from spermatozoa. J. Reprod. Fertil. 1, 310–311.

Brown, D.A., 1993. The tyrosine kinase connection: how GPI-anchored proteins activate T cells. Curr. Opin. Immunol. 5, 349–354.

Bourguignon, L.Y.W., Lokeshwar, V.B., Chen, X., Kerrick, W.G.L., 1993. Hyaluronic acid-induced lymphocyte signal transduction and HA receptor (GP85/CD44)-cytoskelon interaction. J. Immunol. 151, 6634–6644.

Bourguignon, L.Y.W., Zhu, H., Chu, A., Iida, N., Zhang, L., Hung, M.-C., 1997. Interaction between the adhesion receptor, CD44, and the oncogene product, $p185^{HER2}$, promotes human ovarian tumor cell activation. J. Biol. Chem. 272, 27913–27918.

Braithwaite, K.L., Barna, T., Spurway, T.D., 1997. Evidence that galactanase A from *Pseudomonas fluorescens* subspecies cellulose is a retaining family 53 glycosyl hydrolase in which E161 and E270 are the catalytic residues. Biochemistry 36 (15), 489–515, 500.

Brown, D.A., London, E., 1998. Functions of lipid rafts in biological membranes. Ann. Rev. Cell Dev. Biol. 14, 111–136.

Camenisch, T.D., Spicer, A.P., Brehm-Gibson, T. et al., 2000. Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. J. Clin. Invest. 106, 349–360.

Chain, E., Duthie, E.S., 1939. A mucolytic enzyme in testes extract. Nature 144, 977–978.

Chan, C.P., Gupta, S., Mark, G.E., 1999. Identification of linear surface epitopes on guinea pig sperm membrane protein PH-20. Life Sci. 64, 1989–2000.

Cherr, G.N., Yudin, A.I., Katz, D.F., 1990. Organization of the hamster cumulus extracellular matrix: a hyaluronate-glycoprotein gel which modulates sperm access to the oocyte. Dev. Growth Differ. 32, 353–365.

Cherr, G.N., Meyers, S.A., Yudin, A.I. et al., 1996. The PH-20 protein in cynomolgus macaque spermatozoa: Identification of two different forms exhibiting hyaluronidase activity. Dev. Biol. 175, 142–153.

Cherr, G.N., Yudin, A.I., Li, M.-W., Overstreet, J.W., 1999. Hyaluronic acid and the cumulus extracellular matrix induce increases in intracellular calcium in macaque sperm via the plasma membrane protein PH-20. Zygote 7, 211–222.

Cheung, W.F., Cruz, T.F., Turley, E.A., 1999. Receptor for hyaluronan-mediated motility (RHAMM), a hyaladherin that regulates cell responses to growth factors. Biochem. Soc. Trans. 27, 135–142.

Collis, L., Hall, C., Lange, L., Prestwich, G., Turley, E.A., 1998. A rapid hyaluronon uptake is associated with enhanced motility: implications for an intracellular mode of action. FEBS Lett. 440, 444–449.

Coska, A.B., Frost, G.I., Wong, T., Stern, R., 1997. Purification and microsequencing of hyaluronidase isozymes from human urine. FEBS Lett. 417, 307–310.

Csoka, A.B., Scherer, S.W., Stern, R., 1999. Expression analysis of six paralogous human hyaluronidase genes clustered on 3p21 and 7q31. Genomics 60, 356–361.

Delpech, B., Girard, N., Bertrand, P., Courel, M.N., Chauzy, C., Delpech, A., 1997. Hyaluronan: fundamental principles and applications in cancer. J. Intern. Med. 242, 41–48.

Deng, X., Crymmek, K., Martin-DeLeon, P.A., 1999. Biochemical maturation of Spam 1 (PH-20) during epididymal transit of mouse sperm involves modifications of *n*-linked oligosaccharides. Mol. Reprod. Dev. 52, 196–206.

Deng, X., He, Y., Maritn-DeLeon, P.A., 2000. Mouse Spam 1 (PH-20): Evidence for its expression in the epididymis and for a new category of spermatogenic-expressed genes. J. Androl. 21, 822–832.

Drobnis, E.Z., Yudin, A.I., Cherr, G.N., Katz, D.F., 1988. Hamster sperm penetration of the zona pellucida: Kinematic analysis and mechanical implications. Dev. Biol. 130, 311–323.

Eppig, J.J., 1991. Mammalian oocyte development in vivo and in vitro. In: Wassarman, P.M. (Ed.), Elements of Mammalian Fertilization. CRC Press, Boca Raton, pp. 57–76.

Ferguson, M.A.J., 1999. The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. J. Cell Science 112, 2799–2809.

Ferguson, M.A.J., Brimacombe, J.S., Brown, J.R., Crossman, A., Dix, A., Field, R.A., Guther, M.L.S., Milne, K.G., Sharma, D.K., Smith, T.K., 1999. The GPI biosynthetic pathways as a therapeutic target for African sleeping sickness. Biochim. Biophys. Acta 1455, 327–340.

Fitzgerald, K.A., Bowie, A.G., Skeffington, B.S., O'Neill, L.A., 2000. Ras, protein kinase C zet, and I kappa B kinases 1 and 2 are downstream effectors of CD44 during the activation of NF-kappa B by hyaluronic acid fragments in T-24 carcinoma cells. J. Immunol. 163, 2053–2063.

Fivaz, M., Vilbois, F., Pasquali, C., van der Goot, F., 2000. Analysis of glycosyl phosphatidylinositol-anchored proteins by two-dimensional gel electrophoresis. Electrophoresis 21, 3351–3356.

Frost, G.I., Csoka, T., Stern, R., 1997a. The hyaluronidases: A chemical, biological and clinical overview. Trends Glycosci. Glycotech. 8, 419–434.

Frost, G.I., Csoka, T.B., Wong, T., Stern, R., 1997b. Purification, cloning and expression of human plasma hyaluronidase. Biochem. Biophys. Res. Commun. 236, 10–15.

Gaseca, P., Civill, N.D., Harrison, R.A.P., 1994. PH-20 and sperm hyaluronidase: a conceptual conundrum in mammalian fertilization. Biochem. J. 303, 335–336.

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

Harder, T., Scheiffele, P., Verkade, P., Simons, K., 1998. Lipid domain structure of the plasma membrane revealed by patching of membrane components. J Cell Biol. 9, 534–542.

Horejsi, V., Cebecauer, M., Cerny, J., Bridicka, T., Angelisova, P., Drabal, K., 1998. Signal transduction in leucocytes via GPI-anchored proteins: An experimental artefact or an aspect of immunoreceptor function? Immunol. Lett. 63, 63–73.

Petitioner Merck, Ex. 1188, p. 522
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00024

Hou, S.T., Ma, A., Jones, R., Hall, L., 1996. Molecular cloning and characterization of rat sperm surface antigen 2B1, a glycoprotein implicated in sperm-zona binding. Mol. Reprod. Dev. 45, 193–203.

Hunnicutt, G.R., Mahan, K., Lathrop, W.F., Ramarao, C.S., Myles, D.G., Primakoff, P., 1996aa. Structural relationship of sperm soluble hyaluronidase to the sperm membrane protein PH-20. Biol. Reprod. 54, 1343–1349.

Hunnicutt, G.R., Primakoff, P., Myles, D.G., 1996bb. 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. Repod. 55 (1), 80–86.

Ilangumaran, S., Borisch, B., Hoessli, D.C., 1999. Signal transduction via CD44: role of plasma membrane microdomains. Leuk. Lymphoma 35, 455–469.

Iozzo, R.V., Murdoch, A.D., 1996. Proteoglycans of the extracellular environment: clues from the gene and protein side offer novel perspectives in molecular diversity and function. FASEB J. 10, 598–614.

Jones, M.H., Davey, P.M., Aplin, H., Affara, N.A., 1995. Expression analysis genomic structure and mapping to 7q31 of the human sperm adhesion molecule gene SPAM-1. Genomics 29, 196–700.

Kasahara, K., Sania, Y., 2000. Functional roles of glycoshingolipids in signal transduction via lipid rafts. Glycoconjugate J. 17, 153–162.

Kenworthy, A.K., Edinin, M., 1998. Distribution of glycosylphosphotidylinositol-anchored protein at the apical surface of MDCK cells examined at a resolution of < 100 A using imaging fluorescence resonance energy transfer. J. Cell Biol. 142, 69–84.

Kirchhoff, C., Hale, G., 1996. Cell-to-cell transfer of glycosylphosphatidylinositol-anchored membrane proteins during sperm maturation. Mol. Hum. Reprod. 2, 177–184.

Knudson, C.B., Nofal, G.A., Pamintuan, L., Auiar, D.J., 1999. The chondrocyte pericellular matrix: a model for hyaluronan-mediated cell-matrix interactions. Biochem. Soc. Trans. 27, 142–147.

Lathrop, W.F., Carmichael, E.P., Myles, D.G., Primakoff, P., 1990. cDNA cloning reveals the molecular structure of a sperm surface protein, PH-20, involved in sperm-egg adhesion and the wide distribution of its gene among mammals. J. Cell Biol. 111, 2939–2949.

Lepperdinger, G., Müllegger, J., Kreil, G. 2001. Hyal 2-less active, but more versatile? Matrix Biol. (in press).

Lepperdinger, G., Strobl, B., Kreil, G., 1998. HYAL2, a human gene expressed in many cells, encodes a lysosomal hyaluronidase with a novel type of specificity. J. Biol. Chem. 273 (35), 22466–22470.

Lewin, L.M., Nev, Z., Gabsu, A., Weissenberg, R., 1982. The role of sperm bound hyaluronidase in the dispersal of the cumulus oophorus surrounding the rat ova. Int. J. Androl. 5, 37–44.

Li, M., Cherr, G.N., Yudin, A.I., Overstreet., J.W., 1997a. Biochemical characterization of the PH-20 protein on the plasma membrane and using acrosomal membrane of cynomolgus macaque spermatozoa. Mol. Reprod. Dev. 48, 356–366.

Li, M.-W., Yudin, A.I., VandeVoort, C.A., Sabeur, K., Primakoff, P., Overstreet, J.W., 1997b. Inhibition of monkey sperm hyaluronidase activity and heterologous cumuls penetration by flavonoids. Biol. Reprod. 56, 1383–1389.

Lin, Y., Kimmel, L.H., Myles, D.G., Primakoff, P., 1993. Molecular cloning of the human and monkey sperm surface protein PH-20. Proc. Natl. Acad. Sci. USA 90, 10071–10075.

Lin, Y., Mahan, K., Lathrop, W.F., Myles, D.G., Primakoff, P., 1994. A hyaluronidase activity of the sperm membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. J. Cell Biol. 125, 1157–1163.

Li, M-W., Yudin, A.I., Robertson, K.R., Cherr, G.N., Overstreet, J.W. 2001. The Importance of glycosylation and disulfide bonds for the hyaluronidase activity of macaque sperm surface PH-20. J. Androl. (in press).

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

Liu, D., Pearlman, E., Diaconu, E. et al., 1996. Expression of hyaluronidase by tumor cells induces angiogenesis in vivo. Proc. Natl. Acad. Sci. USA 93, 7832–7837.

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

Lokeshwar, V.B., Lokeshwar, B.L., Pham, H.T., Block, N.L., 1996. Association of elevated levels of hyaluronidase, a matrix-degrading enzyme, with prostate cancer progression. Cancer Res. 56 (3), 651–657.

Lokeshwar, V.B., Soloway, M.S., Block, N.L., 1998. Secretion of bladder tumor-derived hyaluronidase activity by invasive bladder tumor cells. Cancer Lett. 131, 21–27.

Lokeshwar, V.B., Young, M.J., Goudarzi, G. et al., 1999. Identification of bladder tumor-derived hyaluronidase: its similarity to HYAL1. Cancer Res. 59, 4464–4470.

Madan, A.K., Yu, K., Dhurandhar, N., Cullinane, C., Pang, Y., Beech, D.J., 1999. Association of hyaluronidase and breast adenocarcinoma invasiveness. Oncol. Rep. 6, 607–609.

Maingonnat, C., Victor, R., Bertrand, P., Courel, M.-N., Maunoury, R., Delpech, B., 1999. Activation and inhibition of human cancer cell hyaluronidase by proteins. Anal. Biochem. 268, 30–34.

Markovic-Housley, Z., Miglierini, G., Soldatova, L., Rizkallah, P.J., Muller, U., Schirmer, T., 2000. Crystal structure of hyaluronidase, a major allergen of bee venom. Structure 8, 1025–1035.

McKee, C.M., Penno, M.B., Cowman, M. et al., 1996. Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages: The role of HA size and CD44. J. Clin. Invest. 98, 2403–2413.

McKee, C.M., Lowenstein, C.J., Horton, M.R., Wu, J., Bao, C., Chin, B.Y., 1997. Hyaluronan fragments induce nitric oxide synthase in murine macrophages through a nuclear factor kappaB-dependent mechanism. J. Biol. Chem. 272, 8013–8018.

Meyer, M.F., Kreil, G., Aschauer, H., 1997. The soluble hyaluronidase from full testes is a fragment of the membrane-bound PH-20 enzyme. FEBS Lett. 413, 385–388.

Meyers, S.A., Yudin, A.I., Cherr, G.N. et al., 1997. Hyaluronidase activity of in vitro capacitated macaque sperm assessed by in vitro cumulus penetration assay. Mol. Reprod. Dev. 46, 392–400.

Minelli, A., Allegrucci, C., Mezzasoma, I., Ronquinst, G., Lluis, C., Franco, R., 1999. CD26 and adenosine deaminase interaction: Its role in the fusion between horse membrane vesicles and spermatozoa. Biol. Reprod. 61, 802–808.

Myles, D.G., Koppel, D.E., Cowan, A.E., Phelps, B.M., Primakoff, P., 1987. Rearrangement of sperm surface antigens prior to fertilization. Ann. NY Acad. Sci. 513, 262–273.

Neame, P.J., Barry, F.P., 1993. The link proteins. Experientia 49, 393–402.

Ohta, K., Sato, C., Matsuda, T. et al., 2000. Co-localization of receptor and transducer proteins in the glycosphingolipid-enriched, low density, detergent-insoluble membrane fraction of sea urchin sperm. Glycoconjugate J. 17, 205–214.

Oliferenko, S., Kaverina, I., Small, J.V., Huber, L.A., 2000. Hyaluronic acid (HA) binding to CD44 activates Rac1 and induces lamellipodia outgrowth. J. Cell Biol. 148 (6), 1159–1164.

Overstreet, J.W., Lin, Y., Yudin, A.I. et al., 1995. Location of the PH-20 protein on acrosome-intact and acrosome-reacted spermatozoa of cynomolgus macaques. Biol. Reprod. 52, 105–114.

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

Petitioner Merck, Ex. 1188, p. 523
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00024

Pham, H.T., Block, N.L., Lokeshwar, V.B., 1997. Tumor-derived hyaluronidase: A diagnostic marker for high-grade bladder cancer. Cancer Res. 57, 778–783.

Phelps, B.M., Myles, D.G., 1987. The guinea pig sperm plasma membrane protein, PH-20, reaches the surface via two transport pathways and becomes localized to a domain after initial uniform distribution. Dev. Biol. 123, 63–72.

Phelps, B.M., Primakoff, P., Chapel, D.E., Low, M.G., Myles, D.G., 1988. Restricted lateral diffusion of PH-20, a GPI-anchored sperm membrane protein. Science 240, 1780–1782.

Phelps, B.M., Koppel, D.E., Primakoff, P., Myles, D.G., 1990. Evidence that proteolysis of the surface is an initial step in the mechanism of formation of sperm cell surface domains. J. Cell Biol. 111, 1839–1847.

Podyma, K.A., Yamagata, S., Sakata, K., Yamagata, T., 1997. Differences of hyaluronidase produced by human tumor cell lines with hyaluronidase present in human serum as revealed by zymography. Biochem. Biophys. Res. Commun. 241, 446–452.

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

Primakoff, P., Cowan, A., Hyatt, H., Tredick-Kline, J., Myles, D.G., 1988. 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. 38, 921–934.

Prinetti, A., Iwabuchi, K., Hakomori, S.-I., 1999. Glycosphingolipid-enriched signaling domain in mouse neuroblastoma Neuro2a cells. J. Biol. Chem. 274 (20), 916–920, 924.

Rai, S.K., Duh, F.-M., Vigdorovich, V., Danilkovitch-Miagkova, A., Lerman, M.I., Miller, A.D., 2001. Candidate tumor suppressor HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for jaagsiekte sheep retrovirus, the envelope protein of which mediates oncogenic transformation. Proc. Natl. Acad. Sci. USA 98 (8), 4443–4448.

Ranganathan, S., Bharadwaj, A., Datta, K., 1995. Hyaluronan mediates sperm motility by enhancing phosphorylation of proteins including hyaluronan binding protein. Cell. Mol. Biol. Res. 41, 467–476.

Rao, C.M., Deb, T.B., Datta, K., 1996. Hyaluronic acid induced hyaluronic acid binding protein phosphorylation and inositol triphosphate formation in lymphocytes. Biochem. Mol. Biol. Int. 40, 327–337.

Robinson, P.J., 1991. Phosphatidylinositol membrane anchors and T-cell activation. Immunol. Today 12, 35–41.

Robinson, P.J., 1997. Signal transduction via GPI-anchored membrane proteins. Adv. Exp. Med. Biol. 419, 365–370.

Sabeur, K., Cherr, G.N., Yudin, A.I., Primakoff, P., Li, M., Overstreet, J.W., 1997. The PH-20 protein in human spermatozoa. J. Androl. 18, 151–158.

Sabeur, K., Cherr, G.N., Yudin, A.I., Overstreet, J.W., 1998. Hyaluronic acid enhances induction of the acrosome reaction of human sperm through interaction with the PH-20 protein. Zygote 6, 103–111.

Saling, P.M., Storey, B.T., 1979. Mouse gamete interactions during fertilization in vitro. Chlorotetracycline as a fluorescent probe for the mouse sperm acrosome reaction. J. Cell Biol. 83, 544–554.

Salustri, A., Camaioni, A., Di Giacomo, M., Fulop, C., Hascall, V.C., 1999. Hyaluronan and proteoglycans in ovarian follicles. Hum, Reprod. Update 5, 293–301.

Simon, K., Ikonian, E., 1997. Sphingolipid-cholesterol rafts in membrane trafficking and signaling. Nature 387, 569–572.

Sobel, M., Soler, D.F., Kermode, J.C., Harris, R.B., 1992. Localization and characterization of a heparin binding domain peptide of human von Willebrand Factor. J. Biol. Chem. 267 (13), 8857–8862.

Stern, R., Shuster, S., Wiley, T.S., Formby, B., 2001. Hyaluronidase can modulate expression of CD44. Exp. Cell Res. 266 (1), 167–176.

Strobl, B., Wechselberger, C., Beier, D.R., Lepperdinger, G., 1998. Structural organization and chromosomal localization of HYAL 2, a gene encoding a lysosomal hyaluronidase. Genomics 53, 214–219.

Struck, D.K., Lennarz, W.J., 1980. The function of saccharide-lipids in synthesis of glycoproteins. In: Lennarz, W.J. (Ed.), The Biochemistry of Glycoproteins and Proteoglycans. Plenum Press, New York, pp. 35–84.

Tamakoshi, K., Kikkawa, F., Maeda, O. et al., 1997. Hyaluronidase activity in gynecological cancer tissues with different metastatic forms. Br. J. Cancer 75, 1807–1811.

Toole, B.P., 1990. Hyaluronan and its binding proteins, the hyaladherins. Curr. Opin. Cell Biol. 2, 839–844.

Toole, B.P., 1997. Hyaluronan in morphogenesis. J. Intern. Med. 242, 35–40.

Toole, B.P., 2000. Hyaluronan is not just a goo! [comment]. J. Clin. Invest. 106, 335–336.

Turley, E.A., 1989. Hyaluronic acid stimulates protein kinase activity in intact cells and in an isolated protein complex. J. Biol. Chem. 264, 8951–8955.

Triggs-Raine, B., Salp, T.J., Zhang, H., Wicklow, B.A., Natowicz, M.R., 1999. Mutations in HYAL 1, a member of tandemly distributed multigene family encoding disparate hyaluronidase activities, cause a newly described lysosomal disorder, mucopolysaccharidosis IX. Proc. Natl. Acad. Sci. USA 96, 6296–6300.

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

VandeVoort, C.A., Cherr, G.N., Overstreet, J.W., 1997bb. Hyaluronic acid enhances the zona pellucida-induced acrosome reaction of macaque sperm. J. Androl. 18, 1–5.

Van Roey, P., Rao, V., Plummer, T.H., Tarentino, A.L., 1994. Crystal structure of endo-β-*N*-acetylglucosaminidase F1, an alpha/beta barrel enzyme adapted for complex substrate. Biochemistry 33 (13), 13,989–13,996.

Varma, R., Mayor, S., 1998. GPI-anchored proteins are organized in submicron domains at the cell surface. Nature 394, 798–801.

Victor, R., Chauzy, C., Girard, N. et al., 1999. Human breast-cancer metastasis formation in a nude-mouse model: studies of hyaluronidase, hyaluronan and hyaluronan-binding sites in metastatic cells. Int. J. Cancer 82, 77–83.

Vines, C.A., Li, M-W., Deng, X., Yudin, A.I., Cherr, G.N., Overstreet, J.W., 2001. Identification of a hyaluronic acid (HA) binding domain in the PH-20 protein that may function in cell signaling. Mol. Reprod. Dev. (in press).

West, D.C., Kumar, S., 1989. The effect of hyaluronate and its oligosaccharides on endothelial cell proliferation and monolayer integrity. Exp. Cell Res. 183, 179–196.

Withers, S.G., Aebersold, R., 1995. Approaches to labeling and identification of active site residues in glycosidases. Protein Sci. 4, 361–372.

Wassarman, P.M., 1990. Profile of a mammalian sperm receptor. Development 108, 1–17.

Yanagimachi, R., 1994. Mammalian fertilization. In: Knobil, E., Neil, J.D. (Eds.), The Physiology of Reproduction, 2nd ed. Raven Press, New York, pp. 189–317.

Yang, B., Zhang, L., Turley, E.A., 1993. Identification of two hyaluron-binding domains in the hyaluron receptor RHAMM. J. Biol. Chem 268 (12), 8617–8623.

Yudin, A.I., Cherr, G.N., Van de Voort, C.A., Overstreet, J.W., 1998. Rearrangement of the PH-20 Protein on the surface of

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macaque spermatozoa following exposure to anti- PH-20 antibodies or binding to zona. Mol. Reprod. Dev. 50, 207–220.
Yudin, A.I., Van de Voort, C.A., Li, M.-W., Overstreet, J.W., 1999. PH-20 but not acrosin is involved in sperm penetration of the macaque zona pellucida. Mol. Reprod. Dev. 53, 350–362.
Yudin, A.I., Li, M.W., Robertson, K.R., Cherr, G.N., Overstreet, J.W., 2001. Characterization of the active site of monkey sperm hyaluronidase. Reproduction 121, 735–743.
Zhang, L., Underhill, C.B., Chen, L., 1995. Hyaluronan on the surface of tumor cells is correlated with metastatic behavior. Cancer Res. 55, 428–433.
Zhang, H. Martin-DeLeon, P.A., 2001. Mouse epidiymal SPAM1 (PH-20) is released in vivo and in vitro, and SPAM1 is differentially regulated in testes and epdidymis. Biol. Reprod. (in press).
Zheng, Y., Deng, X., Zhao, Y., Martin-DeLeon, P.A., 2001. SPAM1 (PH-20) mutations and sperm dysfunction in mice with the Rb (6.16) or Rb (6.15) translocation. Biol. Reprod. (in press).
Zverlov, V., Mahr, S., Riedel, K., Bronnenmeier, K., 1998. Properties and gene structure of a bifunctional celluloytic enzyme (CelA) from the extreme thermophile *Anaerocellum thermophilum* with separate glycosyl hydrolase family 9 and 48 catalytic domains. Microbiology 144, 457–465.

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