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Hyalp1 in Murine Sperm Function: Evidence for Unique and Overlapping Functions With Other Reproductive Hyaluronidases

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ABSTRACT: While Sperm adhesion molecule 1 (SPAM1) is the highly conserved mammalian sperm hyaluronidase (hyase), multiple hyases are present in the mouse testis. In this study we show that one of the murine hyases, Hyalp1, which is predominantly expressed in the testis in a 24-kd isoform has neutral enzymatic activity. On sperm, Hyalp1 is localized on the plasma membrane of the anterior head and was shown to have neutral hyase activity for an isoform of ~55–56 kd, contributing modestly to the overall neutral hyase activity. This activity is associated with in vitro cumulus penetration, since antibody inhibition of Hyalp1 significantly ($P = .034$) retarded the rate of penetration of wild-type (WT) sperm. Antibody-inhibited *Spam1* null sperm were more severely retarded ($P = 4.2 \times 10^{-19}$), suggesting an up-regulation of *Hyalp1* in these mice. A functionality test of the hyaluronic acid (HA) receptor domain identified in the N-terminus by in silico analysis revealed that sperm Hyalp1 is significantly ($P = .006$) involved in the progesterone-induced HA-enhanced acrosome reaction. Finally, developmental reverse transcription polymerase chain reaction (RT-PCR) shows that testicular transcripts of *Hyalp1* are detected as early as 6 days postparturition, similar to transcripts for *Spam1,* suggesting that the gene might also play a role in the developing testes prior to spermiogenesis. Taken together, the findings reveal that Hyalp1 likely has a unique function in the adult testis, and redundant overlapping ones with Spam1 and may compensate for it in *Spam1* null mice.

Key words: Sperm antigen, hyaluronidase activity, *Spam1* null sperm, acrosome reaction, cumulus penetration, hyaluronic acid receptor.

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The unfertilized oocyte is surrounded by several formidable barriers: the outer cumulus, zona pellucida (ZP), and perivitelline space. These barriers are rich in hyaluronic acid (HA), a repeated glycosaminoglycan. The digestion of HA and subsequent penetration of these barriers which are required for fertilization have generally been thought to be due to hyases (Talbot, 1984; Zaneveld and DeJonge, 1991; Dandekar and Talbot, 1992). Sperm adhesion molecule 1 (SPAM1 or PH-20), which is widely conserved in mammals (Lathrop et al, 1990), is the major sperm hyase (Lin et al, 1994; Myles and Primakoff, 1997). SPAM1 is encoded by a gene on human 7q31 (*SPAM1*; Jones et al, 1995) and on mouse 6A2 (*Spam1*; Deng et al, 1997) and has been extensively characterized in mammals. *SPAM1*/*Spam1* resides in a gene cluster, the reproductive hyases, with 2 related murine family members, *Hyal5* and *Hyalp1,* which have no functional human homologs (Csoka et al, 1999). Recently, both *Hyal5* and *Hyalp1* were shown to be present in murine sperm (Zhang et al, 2005), and *Hyal5* was reported to have hyase activity (Kim et al, 2005). However, the function(s) of *Hyalp1* and whether or not it interplays with *Spam1* are not yet understood.

In addition to hyase activity, SPAM1 has been shown to perform other roles in fertilization, including ZP binding and acrosomal exocytosis (Hunnicutt et al, 1996; Sabeur et al, 1998; Cherr et al, 1999; Vines et al, 2001). However, sperm from *Spam1* null mice are fertile with a delay in cumulus penetration, indicating functional compensation for a lack of *Spam1* by other proteins (Baba et al, 2002). *Hyalp1* and *Hyal5* are conspicuous candidates for this position because of strong sequence homology and similarity of domains with *Spam1* (Zhang et al, 2005). Further, the Rb(6.16) and Rb(6.15) Robertsonian translocations, which were shown to bear point mutations of *Spam1* and to be associated with sperm dysfunction in homozygotes, as well as transmission ratio distortions (TRDs) in the heterozygotes (Zheng and Martin-DeLeon, 1997; Zheng et al 2001a,b), were recently shown to also carry mutations in *Hyalp1* and *Hyal5* (Zhang et al, 2005). Thus, mutations in each of these 3 hyases seemingly contribute to the TRDs. Interestingly, attempts at overexpression of both *Spam1* and *Hyal5* have met with strong resistance, suggesting stringent regulatory control of the reproductive hyases (Zhang et al, 2006).

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Four separate domains for functional activity exist within SPAM1 and account for each of its independent roles in fertilization: neutral and acidic hyaluronidase activity (Cherr et al, 2001), HA-binding (Sabeur et al, 1998; Morales et al, 2004; Zhang et al, 2005), and ZP-binding (Myles and Primakoff, 1997; Cherr et al, 2001). Neutral enzymatic activity, a characteristic unique to the reproductive hyases, dominates the membrane-bound SPAM1 and is responsible for penetration of the cumulus cell-oocyte matrix. Second, soluble SPAM1 cleaved during the acrosome reaction is characterized by acidic hyase activity and is responsible for digestion of hyaluronan or HA in the ZP (Hunnicutt et al, 1996; Cherr et al, 2001). A hyaluronan-binding domain exists in SPAM1 separately from either hyase domain and contributes to the acrosome reaction signaling pathway via a HA-enhanced increase in Ca^{2+} influx after the acrosome reaction (Sabeur et al, 1998; Cherr et al, 1999; Vines et al, 2001). The signal transduction of the acrosome reaction, required for the release of soluble enzymes that aid in the penetration of the ZP, has been linked to SPAM1 in humans (Cherr et al, 2001) and mice (Morales et al, 2004). Fourth, a ZP binding domain in the carboxy-terminal end of SPAM1 facilitates secondary ZP binding after the acrosome reaction (Hunnicutt et al, 1996; Myles and Primakoff, 1997).

While *HYALP1* is a pseudogene in humans (Csoka et al, 1999), its RNA and protein are abundantly expressed in the testis of the mouse (Zhang et al, 2005), where it is located ~27.2 kb upstream of *Spam1*. However, apart from a knowledge of the location of the murine gene (*Hyalp1*) and the size of the mRNA and protein (Zhang et al, 2005), nothing has been published on its functional role. This investigation thus focuses on the expression and physiological role of *Hyalp1* in murine reproduction and its potential to compensate for a lack of functional *Spam1*. Our results reveal a possible unique role for Hyalp1 in the testis as well as in sperm, where it has a functional overlap with the well-characterized Spam1. Importantly, the data suggest a cross-talk between the encoding genes.

Materials and Methods

In Silico Analyses

Hyalp1 sequences used in all analyses were obtained from GenBank (accession number AK016575). Hyalp1 protein was aligned with both Hyal5 and Spam1 proteins translated from the cDNAs (GenBank accession numbers AK017112 and AK005638, respectively) using ClustalW (European Bioinformatics Institute, Cambridge, United Kingdom) and color-coding of physiochemically similar residues was superimposed. Putative domains for enzymatic activity of Spam1 (Cherr et al, 2001) were highlighted and compared to the Hyalp1 sequence for identity.

Animals and Reagents

Sexually mature ICR or C57BL/6 (Harlan Sprague-Dawley, Indianapolis, Ind) mice were used in all studies except developmental reverse transcription polymerase chain reaction (RT-PCR), where ages are indicated. Studies were approved by the Institutional Animal Care and Use Committee at the University of Delaware and conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication 85–23, revised 1985). *Spam1* null mice on the ICR background were obtained from the laboratory of Tadashi Baba in Japan. All chemicals were purchased from Sigma Chemical Company (St Louis, Mo) or Fisher Scientific Company (Malvern, Pa), unless otherwise specified.

Development of Hyalp1 Antibody

A polyclonal anti-peptide (Zymed Laboratories, South San Francisco, Calif) was created for a unique C-terminus sequence for Hyalp1 (GenBank accession number AK106575) by way of a PolyQuik rabbit antiserum. C-terminus specificity was assessed by competing with the peptide (amino acid residues 477–494: C YNGNFSLKPLKR-REIIFL) used in development of anti-serum, which has been previously characterized (Zhang et al, 2005).

Protein Extraction From Whole Testicular Tissue and Sperm

This was performed as described in Zhang et al (2004). Briefly, whole testicular tissue was manually homogenized (using a mortar and pestle) with a solubilization buffer (62.5 mM Tris-HCl, 10% glycerol, 1% SDS, pH 6.8) containing a protease inhibitor, 1% phenylmethylsulfonyl fluoride (PMSF) at 4°C. The suspension was then centrifuged at 12 000 × *g* at 4°C for 15 minutes and the supernatant collected. Cauda sperm were collected from minced epididymides and incubated in 5 mL sperm suspension buffer (50 mM Tris, 20 mM EDTA containing 1% PMSF at 37°C) to disperse sperm and allow them to swim out. Tissues were pelleted at 500 × *g* for 2 minutes and sperm collected from the suspension by centrifugation at 1000 × *g* for 10 minutes at 4°C. Protein extracts were prepared by lysing sperm with solubilization buffer as above and the suspension vigorously vortexed for 4 minutes prior to centrifugation. The supernatants from testis and sperm were collected and protein concentrations measured using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, Ill), using different concentrations of BSA as standards.

HA Substrate Gel Electrophoresis

Hyase activity in testicular and sperm protein extracts was measured using HA substrate gel electrophoresis (HASGE), as described by Guntenhoner et al (1992) and Deng et al (2000). HA from bovine vitreous humor or human umbilical cord was added to a 15% (10% in the case of sperm protein) SDS-

polyacrylamide gel (final concentration 0.15–0.3 μg/mL). Approximately 30 minutes before the gels were loaded, the protein samples were treated with either 1:10 dilution of preimmune serum or Hyalp1 antiserum. Gels were loaded with 60 μg protein for the testis or 6 μg for sperm and run at 15 mA. BSA negative controls were also loaded. After completion of electrophoresis, gels were incubated in 3% TritonX-100 in PBS for 2 hours at RT, then at 37°C for 24 to 36 hours in 100 mM sodium acetate (pH 7.0). To visualize digestion of HA, gels were stained with 0.5% alcian blue in 3% acetic acid for 2 hours, and then destained in 7% acetic acid until digestion was visible. Gels were counterstained with Coomassie Brilliant Blue G-250 and destained with methanol-acetic acid and scanned to image the bands.

Immunocytochemistry of Epididymal Sperm

To visualize the localization of Hyalp1 relevant to cumulus penetration (ie, protein available for activity on the sperm surface), immature and mature sperm were collected in PBS by mincing caput and cauda epididymides and allowing sperm to float or swim out, respectively, after slight agitation of the dish. For caput epididymides, mincing tissues allows sperm to disperse in the PBS, albeit at a lower concentration than that for motile sperm, which are able to swim. However, adequate numbers of caput sperm were obtained for immunocytochemistry in each trial using this method. Briefly, sperm were washed and blocked for 30 minutes in 3% bovine serum albumin (BSA) and 1% normal donkey serum in PBS. Sperm were incubated overnight at 4°C in rabbit polyclonal antibody against mouse Hyalp1, then for 30 minutes at RT in FITC-labeled donkey anti-rabbit IgG. Preimmune rabbit serum was used in place of Hyalp1 antibody as a control. Sperm were then mounted in p-phenylenediamine antifade with 1.5 μg/mL of 4′ 6-diamidino-2-phenylinodole (DAPI) and viewed on a Zeiss Axiophot fluorescence microscope (Thornwood, NY) using the appropriate FITC or DAPI filter set. Imaging was done using a CCD-cooled camera.

In Vitro Fertilization Vestment Penetration

Female ICR mice 8–10 weeks old were superovulated using successive intraperitoneal injections of pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) 48 hours apart (7.5 IU each). Eggs were collected from oviductal ampulla 13.5–14 hours after HCG injection and placed in 1 mL human tubal fluid (HTF), covered with mineral oil, and equilibrated at 37°C under 5% CO_2, 5% O_2, 90% N_2. Fresh caudal epididymal sperm from both WT (*Spam1* +/+) sperm and sperm lacking functional Spam1 (*Spam1* −/−) were capacitated for 30–45 minutes in 1 mL HTF under the same conditions, as described by Chen et al (2006). Capacitated sperm (7.5×10^4) were added to treatment dishes containing 0.5 mL HTF and either preimmune serum or Hyalp1 antiserum (1:5) for 30 minutes. Eggs were then incubated with sperm, and individual eggs were scored at 20-minute intervals (total 6 hours) for the degree of cumulus penetration.

Assessment of penetration through the cumulus of oocytes was similar to that of Baba et al (2002). Briefly, the progress of cumulus cell-removal was categorized in 4 stages (1–4), based on the appearance of the cumulus masses after microscopic observation. Stage 1 had eggs with tightly packed cumulus cells (such that the eggs were fully occluded); Stage 2 had cumulus masses in which the eggs could be visualized with loosely associated cumulus cells; Stage 3 had eggs in which most of the cumulus cells had been dispersed from the masses; and Stage 4 had eggs with complete dispersal of the cumulus cells. The data were subjected to statistical analysis, using Fisher's exact test, to determine significant differences in the number of eggs penetrated for the different treatments.

Progesterone Enhanced Acrosome Reaction Assay

Induction of acrosome reactions was similar to that of Sabeur et al (1998) and Morales et al (2004). Briefly, fresh cauda epididymal sperm (2.5×10^5) from age-matched sexually mature WT ICR males were suspended in Biggers Whitten Whittingham (BWW) medium and then treated under several conditions. Controls were treated with 0.1% DMSO in BWW-Hepes for 5 minutes. Preimmune serum or Hyalp1 antiserum (1:50) was incubated for 10 minutes at RT. Samples treated with HA (100 μg/mL) for 30 minutes were successively treated with progesterone (PG; 3.18 μM) for 5 minutes before fixation with 4% paraformaldehyde. Slides were prepared, stained with 0.44% Coomassie Brilliant Blue G-250 in 60% methanol-acetic acid, rinsed in ddH_2O, and sealed with Permount. Slides were microscopically analyzed, and at least 200 sperm were scored for the presence (+) or absence (–) of the acrosome cap, as previously described (Morales et al, 2004). The results were subjected to a Kruskal-Wallis one-way ANOVA on ranks for statistical analysis.

Reverse Transcriptase-PCR

Total testicular RNA was extracted from whole testis using Trizol reagent according to manufacturers protocol, and samples were treated with RNase-free DNase (final concentration 5–10 U/mL; Boehringer Mannheim, Basel, Switzerland) followed by phenol/chloroform extraction. Reverse transcriptase control (−) and test (+) samples were prepared using 2 μL of RNA extract with a SuperScript Preamplification system. Amplification was carried out using a forward primer (nts 1976–1999) 5′ agg cat tag gaa gta cta gag agc 3′ and reverse primer (nts 2288–2305) 5′tct tct ggg ttt gct cct 3′. PCR was performed under the following conditions: 35 cycles of 94°C for 3 minutes, 54°C for 2 minutes, 72°C for 12 minutes. PCR products were resolved on a 1% agarose gel stained with ethidium bromide.

Results

In silico analysis revealed that murine *Hyalp1* has an average structural similarity (homology) of 60% with the 2 other reproductive hyases at the nucleotide level. The resultant protein shows 41% homology with both Spam1 and Hyal5, and ClustalW alignments (Figure 2b; Zhang et al, 2005) reveal extensive physiochemical

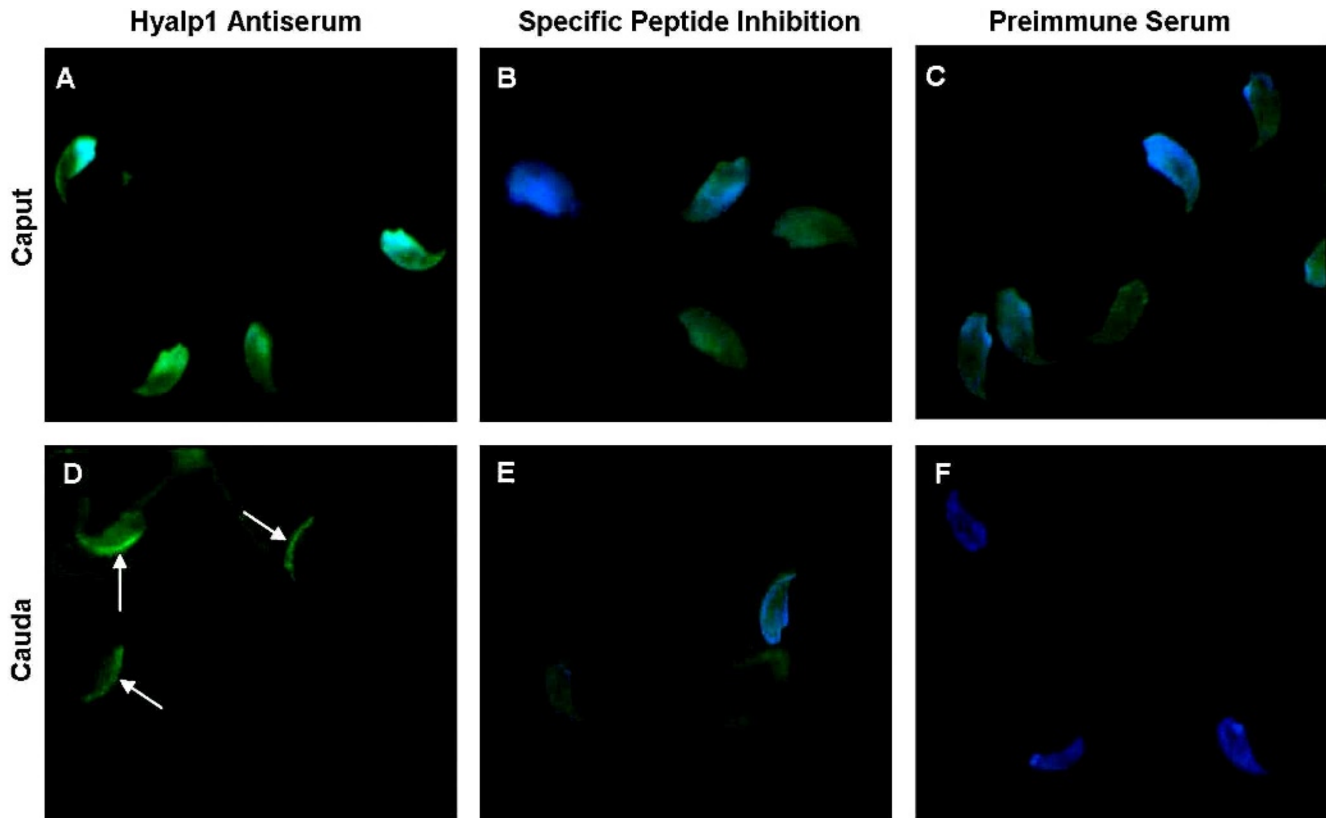

Figure 1. Immunocytochemical analysis of caput and caudal epididymal sperm. Treatment with Hyalp1 antiserum and FITC-conjugated secondary antibody reveals a distinct localization of Hyalp1 on the acrosome cap in mature caudal sperm **(D)** and a more dispersed expression in caput sperm **(A)**. Control samples, treated with Hyalp1 peptide-inhibited antiserum **(B, E)** and preimmune serum **(C, F)**, indicate antibody specificity. Unfixed sperm were counterstained with DAPI and viewed on a fluorescence microscope.

similarity with all reproductive hyases. Various analyses have identified 3 exons in *Hyalp1*, 3 potential *N*-linked glycosylation sites in Hyalp1 at amino acid residues 106, 154, and 189. No *O*-linked glycosylation sites were identified, but several potential phosphorylation sites, Ck2, PKC, and Tyr, were found. Importantly, a glycosyl phosphatidylinositol (GPI) anchor was predicted near the C-terminus. Notably absent was a CRE (cyclic AMP response element) in the upstream promoter region of *Hyalp1,* elements that are present in the promoters of *Spam1* and *Hyal5* and other predominantly haploid-expressed spermatogenic genes (Delmas et al, 1993).

Although Spam1 and Hyalp1 are ~41% homologous along the length of the proteins, homology and identity in the putative regions for enzymatic activity are considerably greater than that along the entirety of the proteins. Further examination of ClustalW alignment of the reproductive hyases (Zhang et al, 2005) revealed that Spam1 and Hyalp1 are 29% identical along the length of the proteins, but within the neutral hyase domain of Spam1, this identity increases to 49%. The 2 proteins have the highest identity, 59%, in the region for HA-binding receptor. There is 41% identity in the acidic hyase domain and 54% in the ZP binding domain. These high levels of identity within the functional domains suggest important physiological roles for Hyalp1 in fertilization.

Immunocytochemical analysis reveals the presence of Hyalp1 on the surface of both caput (immature) and caudal (mature) epididymal sperm (Figure 1). On caput sperm the protein is dispersed on the head, with heavier deposits near the posterior. However, it is very distinctly localized to the acrosome cap of caudal sperm. This localization is similar to that of membrane-bound Spam1 (Deng et al, 1999) and implies functionality on the sperm surface before the acrosome reaction, that is, in cumulus penetration. Both preimmune serum and Hyalp1 peptide-inhibited antiserum served as controls and assured antibody specificity, as treatment of sperm under these conditions did not produce specific staining.

For sperm where Western analysis showed 2 Hyalp1 isoforms of ~40 and 66 kd (Zhang et al, 2005), HASGE assays revealed that the bands for all the murine hyases comigrate (Figure 2A), as previously shown (Deng et al,

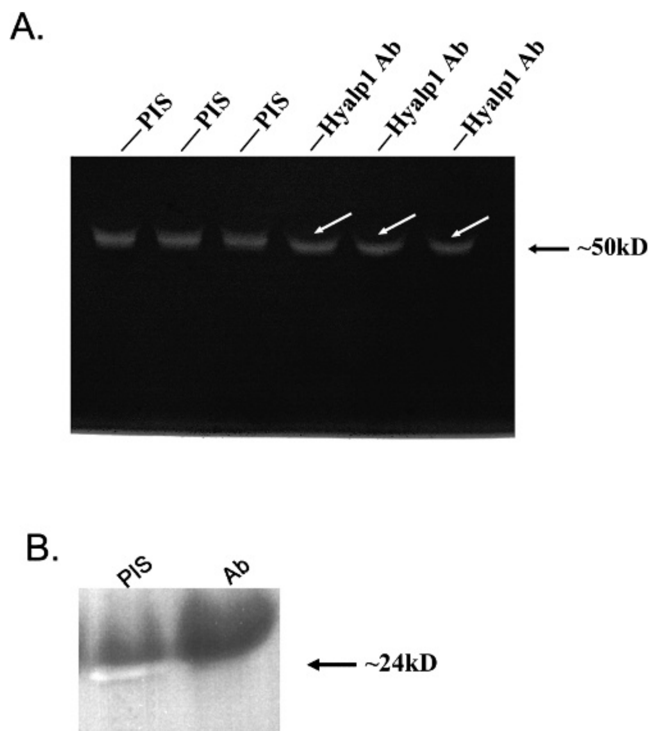

Figure 2. HASGE showing neutral hyase activity of Hyalp1 in testis and sperm. Proteins from testis (60 µg) and sperm (6 µg) were loaded for each sample pretreated with either preimmune serum (PIS) or Hyalp1 antiserum (Ab). **(A)** For sperm, the 3 lanes on the left with proteins treated with PIS show comigrated bands that are broader than those treated with Hyalp1 antiserum on the right. The area of the band reduction with the antiserum is arrowed. **(B)** In the testis there was prominent hyase activity at 56–65 kd and comigration and swirling of the bands, but no differences were detected between the lanes treated with antiserum and with PIS (data not shown). However, at the bottom of the gel (shown here) at ~24 kd in lane 1 a clear band representing the digestion of HA can be seen in the protein treated with PIS. The disappearance of this distinct band with the addition of the Ab illustrates that the ~24-kd isoform is enzymatically active at pH 7.0. The staining above the clear HASGE band is Coomassie Brilliant Blue staining of testis proteins.

2000). In Figure 2A, the addition of Hyalp1-specific antiserum slightly diminished the overall hyase activity in the comigrated bands compared to those samples that were treated with preimmune serum. The missing region of the comigrated bands was in the range ~55–56 kd, suggesting that the neutral hyase activity of Hyalp1 results from an isoform of this MW. Based on the size of the missing region, it appears that Hyalp1 contributes only modestly to the overall neutral hyase activity.

Previous studies have shown via Western blots (Zhang et al, 2005) that the predominant isoform of Hyalp1 in the testis is a 24-kd protein, and HASGE analysis in this study indicates that this isoform is enzymatically active. HASGE assays showed the disappearance of a single distinct clear band of ~24 kd at pH 7.0 when testicular proteins were treated with Hyalp1 antiserum (Figure 2B). The clear band seen in the protein sample treated with preimmune serum demonstrates hyase activity, and the identity of the contributing protein is confirmed by its disappearance with the addition of Hyalp1-specific antiserum. Other clear digestion bands with higher MWs were not noticeably affected by the addition of Hyalp1 antiserum. BSA controls revealed no hyase activity (data not shown).

A total of 389 cumulus oocyte complexes were examined in 4 groups for sperm penetration. Groups 1 and 2 were incubated with WT sperm treated or untreated with Hyalp1 antiserum, while groups 3 and 4 were *Spam1* null sperm similarly treated and incubated. No significant difference was observed between sperm incubated with and without preimmune serum in HTF (data not shown). However, antibody inhibition of Hyalp1 on WT (*Spam1* +/+) sperm was shown to retard cumulus penetration through all 4 stages (Figure 3Aa and b). A Fisher's exact test indicates statistical differences ($P = .034$) between the cumulus penetration of antibody inhibited and noninhibited WT sperm after 6 hours of incubation (Figure 3B).

Performance of the vestment penetration assay using sperm from *Spam1* null mutants in which Hyalp1 is antibody-inhibited, essentially "double null" sperm, reveals a markedly strong inability to penetrate the cumulus when compared to their similarly treated WT (*Spam1* +/+) counterparts (Figure 3Ac and d). Less than 10% of all oocytes reach the final stage of cumulus penetration, after 6 hours of incubation. A Fisher's exact test revealed a highly significant statistical difference ($P = 4.2 \times 10^{-19}$) between the cumulus penetration of Hyalp1 antibody-inhibited and noninhibited *Spam1* null sperm (Figure 3B). While a statistical difference exists between antibody-inhibited and noninhibited sperm from *Spam1* +/+ mice, the difference is much less pronounced. Qualitatively, the impediment of cumulus penetration is most apparent with the retarded progression of cumulus penetration between 1 and 5 hours of incubation with sperm from *Spam1* +/+ males.

To test the functionality of the HA-binding domain on Hyalp1, mature WT sperm were subjected to a progesterone-induced HA-enhanced acrosome reaction assay. Progesterone increased the rate of acrosome reaction in the presence of HA ($P = .005$); however, the addition of Hyalp1 antiserum significantly reduced the rate of acrosome reaction despite treatment with HA and PG ($P = .006$), as seen in Figure 4. Thus the HA receptor on Hyalp1 is functional and contributes to the HA-binding activity that induces acrosomal exocytosis.

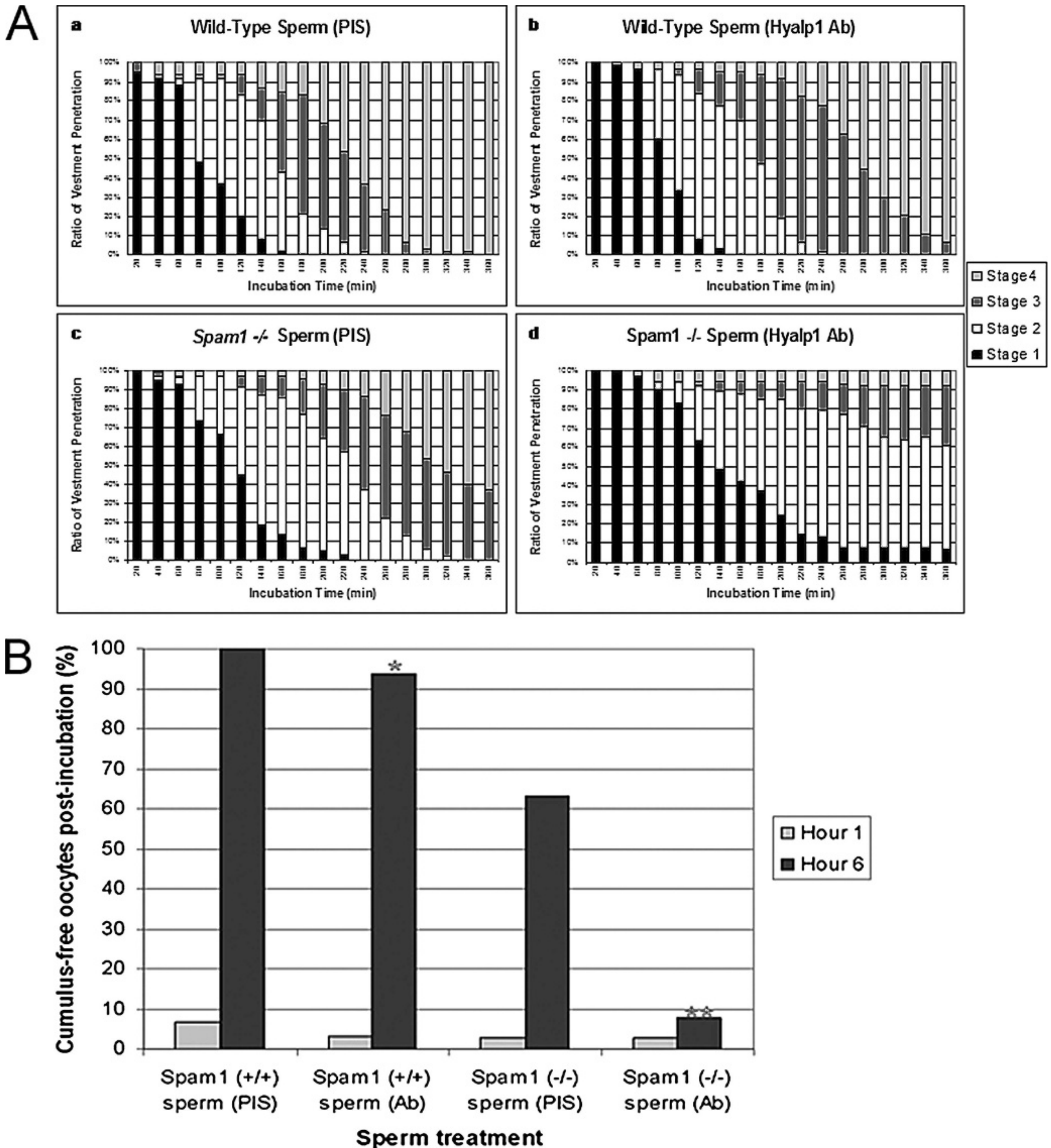

Figure 3. **(A)** Rate of cumulus penetration by WT and *Spam1* null sperm incubated with Hyalp1 preimmune serum and antiserum. Penetration by WT sperm **(a)** is retarded when Hyalp1 is antibody-inhibited **(b)**. Antibody-inhibition of Hyalp1 on null sperm **(d)** causes a more dramatic inhibition of penetration than WT sperm when compared to the preimmune serum (PIS) control **(c)**. A total of 90, 93, 103, and 103 eggs were examined for *Spam1* +/+ (Hyalp1 PIS), *Spam1* +/+ (Hyalp1 Ab), *Spam1* −/− (Hyalp1 PIS), and *Spam1* −/− (Hyalp1 Ab) treatments, respectively. **(B)** Penetration of Hyalp1 antibody-inhibited sperm at 1 and 6 hours after incubation. Inhibition of Hyalp1 significantly retards cumulus penetration with sperm from both *wild-type ($P = .034$) and ***Spam1* −/− ($P = 4.2 \times 10^{-19}$) males. A Fisher's exact test was used for statistical analysis.

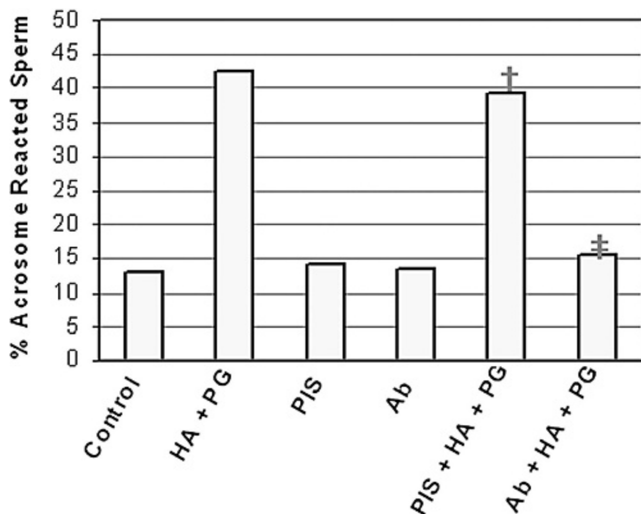

Figure 4. Rate of acrosome reaction with different treatments. Control sperm (no treatment) do not differ in the rates of acrosome reaction with sperm incubated in Hyalp1 preimmune serum (PIS) or antiserum (Ab). The successive addition of HA and progesterone (PG) significantly raises the rate of acrosome reaction, compared to control (P = .005), and addition of PIS has no effect on the increase (†P = .007), but antibody-inhibition of Hyalp1 significantly reduces the rate of acrosome reaction in the presence of HA and PG (‡P = .006) (Kruskal-Wallis one-way ANOVA on ranks).

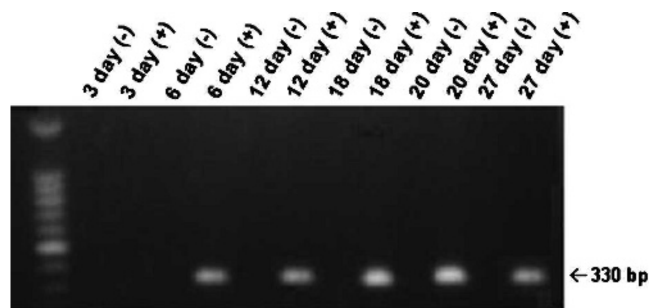

Figure 5. Developmental RT-PCR for the detection of *Hyalp1* RNA. RNA transcripts can be detected in the testes of 6-day mice, but not as early as 3 days postparturition. Total RNAs were reverse transcribed with RT (-) controls. The gel is representative of 3 independent repetitions for each stage of development.

Testicular *Hyalp1* transcripts were detected as early as 6 days postparturition, but not in the testis of 3-day neonates (Figure 5). The negative PCR result at 3 days was not due to the quality of the RNA or PCR failure, as PCR on the identical cDNA samples revealed the presence of transcripts for *Hyal3* (K. Reese, personal communication), a somatic hyase on chromosome 9 (Csoka et al, 1999). Developmental RT-PCR reveals that expression of *Hyalp1* RNA continues through adulthood. Though seeming to be expressed earlier than *Spam1* and *Hyal5*, which by Northern analysis were previously reported to first appear at 21 days (Zheng et al, 2001b; Kim et al, 2005), re-evaluation of the expression of these hyases revealed that both their RNA and protein can be detected in the testis of 6-day neonates by RT-PCR and Western blot, respectively (data unpublished). Thus the 3 reproductive hyases are expressed in early testis development.

Discussion

Four functional domains for activity required for fertilization have been identified in Spam1, though a lack of the protein does not render null mutants infertile (Baba et al, 2002). In initial evaluation of the potential compensation of Hyalp1 for Spam1, alignments of proteomic sequences revealed significant physiochemical similarity between the 2 reproductive hyaluronidases. The increase in both homology and identity (41%–59%) between the proteins within the putative regions of functional activity for Spam1 provides evidence for functional roles of Hyalp1 in fertilization, though each of these suspected roles must be assessed independently. While the predicted GPI anchor uncovered is consistent with the functional roles of Hyalp1 and its redundancy with Spam1, the lack of a CRE element in *Hyalp1* promoter as seen for *Spam1* (Zheng and Martin-DeLeon, 1999) suggests differences in the regulation of these genes. A CRE element is generally associated with abundantly haploid-expressed genes (Delmas et al, 1993), and thus it is likely that in spermatids *Hyalp1* RNA may be less abundant than *Spam1*. Importantly, the most abundant Hyalp1 testicular isoform, the 24-kd protein, is negligibly present on sperm, consistent with the idea that the gene is more abundantly expressed in diploid testicular cells.

Immunocytochemical analysis in this study confirms the finding from Western analysis that the protein is present on sperm (Zheng et al, 2005), and localizes it to the acrosome cap of mature cells. The presence of Hyalp1 in a more uniform distribution on immature caput sperm and its localization on caudal sperm is similar to Spam1 (Deng et al, 1999) and suggests that it undergoes reorganization on the sperm surface during epididymal maturation, similar to Spam1. In this regard it should be mentioned that, similar to Spam1 (Martin-DeLeon, 2006), Hyalp1 has been detected in sperm-free epididymal tissues and epididymal luminal fluid (unpublished data).

At pH 7.0, hyase activity of Hyalp1 was shown for an ~55–56-kd isoform in sperm, as there was a detectable reduction of the comigrated HASGE bands in this region in the presence of Hyalp1 antiserum. This isoform is smaller than the ~66-kd protein found in

mature sperm (Figure 2A; Zhang et al, 2005), so it is likely that hyase activity of Hyalp1 results from processing of a larger isoform either by deglycosylation or endoproteolytic cleavage, as seen for Spam1 (Deng et al, 1999). Neutral hyase activity on mouse sperm has been shown to occur for a 52-kd Spam1 isoform (Baba et al, 2002) and a 55-kd Hyal5 isoform (Kim et al, 2005), and we have reported these sperm proteins to be 67 and 66 kd, respectively (Deng et al, 1999, Zhang et al, 2005). Since neutral hyase activity is unique to the reproductive hyases (Csoka et al 1999), the finding of an ~55–56-kd Hyalp1 isoform with neutral hyase activity supports the inclusion of Hyalp1 in this category of sperm proteins. It also indicates that sperm hyase activity in the mouse is controlled by at least 3 genes. In testicular proteins where the 24-kd isoform of Hyalp1 was the only detectable digestion band to disappear with Hyalp1 antiserum inhibition, there was no other hyase activity from proteins of a similar MW. This suggests that hyase activity of this Hyalp1 isoform may be unique in the testis.

Vestment penetration assays conducted in vitro illustrate that the neutral hyase activity detected for Hyalp1 plays a role in the penetration of the cumulus oocyte complex. Antibody-inhibition of Hyalp1 on mature sperm significantly retarded cumulus penetration on WT sperm, which is consistent with its hyase activity. While the difference between penetration by WT and *Spam1* null sperm (~60%; Figure 3B) was similar to that reported by Baba et al (2002), penetration for null sperm was more significantly retarded by Hyalp1 antibody-inhibition than in WT sperm. The data therefore suggest an up-regulation of *Hyalp1* in *Spam1* null sperm, which were more severely inhibited by Hyalp1 antiserum.

In *Spam1* null mice, up-regulation of *Hyalp1* would increase the contribution of Hyalp1, which would thus constitute proportionately more of the total sperm hyases than present in WT sperm. When Hyalp1 is antibody-inhibited, effectively creating "double null" sperm, cumulus penetration is severely impeded. This could result if Hyalp1 is a major contributor in the absence of Spam1, and strongly suggests compensatory activity of this hyase. The failure of complete blockage of cumulus penetration in the "double null" sperm argues for the presence of other functional sperm hyase(s). Recently, Hyal5 was shown to be involved in murine sperm penetration through the cumulus (Kim et al, 2005). However, Kim et al (2005) concluded that mouse epididymal sperm contain only 2 hyaluronan-hydrolyzing proteins, Hyal5 and Spam1 (PH-20). Our previously published work (Zhang et al, 2005) and the results in the present study argue cogently against this and show that Hyalp1 contributes to hyaluronan-hydrolyzing activity on sperm. Importantly, our findings make a strong case that hyaluronan-hydrolyzing activity of the extracellular matrix of the cumulus is a polygenic trait in the mouse.

It is important to consider the role that may be played by the predominant ~40-kd Hyalp1 sperm isoform that is not involved in hyaluronan-hydrolyzing activity. Kim et al (2005), using biotinylated ZP, showed that the ZP-binding domain of Spam1 and Hyal5 functions very little in sperm-egg interaction. We speculate that the predominant ~40-kd Hyalp1 isoform on sperm may function in this capacity and may be the major ZP-binding hyase.

Hyalp1 could also have compensatory effects with respect to the induction of the acrosome reaction. Binding of SPAM1/Spam1 to HA, via its receptor, in the presence of progesterone in the uterine environment induces acrosomal exocytosis and has been demonstrated in vitro (Sabeur et al, 1998; Morales et al, 2004). In silico analysis indicates that Hyalp1 retains 59% identity with Spam1 in this HA-binding domain, the highest identity for the functional domains. The significant reduction in the rate of acrosome reaction for WT sperm after incubation with Hyalp1 antiserum indicates the involvement of Hyalp1 in the acrosome reaction signaling pathway in WT mice, which likely acts by the same mechanism as does that for Spam1. However, further investigation is required to implicate the involvement of Hyalp1 in compensation for Spam1 with respect to HA-binding.

Taken together, the data reveal that Spam1 and Hyalp1 have redundant, overlapping functions. Hyalp1 should therefore be considered a likely candidate in the compensation for Spam1 in *Spam1* null sperm. The discovery of Hyalp1 functionality does not detract from the potential for additional compensatory or perhaps even cooperative activity of other proteins in Spam1 null mutants. With the knowledge that Hyalp1 can compensate for Spam1 physiologically, it is important to investigate the mechanism of gene control and cross-talk between the reproductive hyases.

Finally, early detection of *Hyalp1* RNA in the testis of 6-day postnatal mice indicates for the first time a role for the reproductive hyases in the premeiotic stage of spermatogenesis. Further evaluation of *Spam1* and *Hyal5,* which had previously been studied only with Northern analysis (Zheng and Martin-DeLeon, 1997; Kim et al, 2005), revealed a similar early expression of transcripts using RT-PCR, as well as the proteins (our unpublished data). This finding suggests a potentially novel role for the proteins of this gene family during spermatogenesis. Germ cells begin migration through the testis at birth; at 4–6 days postparturition, these germ cells implant into the basement membrane of the

seminiferous tubules and are capable of differentiation into type A spermatogonial stem cells (McLean et al, 2003). While *Hyalp1* RNA is undetected in 3-day testes, suggesting that the protein might not be involved in the migration of the gonocytes from the center of the seminiferous tubule to the basement membrane, its early expression at 6 days suggests that it might be present in type A spermatogonia and may play a possible role in the initiation or progression of spermatogenesis. Further studies are underway to investigate the role of this protein in the testis during spermatogenesis.

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