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# Hyaluronidase 2: A Novel Germ Cell Hyaluronidase with Epididymal Expression and Functional Roles in Mammalian Sperm[1]

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## ABSTRACT

**To initiate the crucial cell adhesion events necessary for fertilization, sperm must penetrate extracellular matrix barriers containing hyaluronic acid (HA), a task thought to be accomplished by neutral-active hyaluronidases. Here we report that the ~57 kDa hyaluronidase 2 (HYAL2) that in somatic tissues has been highly characterized to be acid-active is present in mouse and human sperm, as detected by Western blot, flow cytometric, and immunoprecipitation assays. Immunofluorescence revealed its presence on the plasma membrane over the acrosome, the midpiece, and proximal principal piece in mice where protein fractionation demonstrated a differential distribution in subcellular compartments. It is significantly more abundant in the acrosome-reacted ($P = 0.04$) and soluble acrosomal fractions ($P = 0.006$) (microenvironments where acid-active hyaluronidases function) compared to that of the plasma membrane where neutral hyaluronidases mediate cumulus penetration. Using HA substrate gel electrophoresis, immunoprecipitated HYAL 2 was shown to have catalytic activity at pH 4.0. Colocalization and coimmunoprecipitation assays reveal that HYAL2 is associated with its cofactor, CD44, consistent with CD44-dependent HYAL2 activity. HYAL2 is also present throughout the epididymis, where *Hyal2* transcripts were detected, and in the epididymal luminal fluids. In vitro assays demonstrated that HYAL2 can be acquired on the sperm membrane from epididymal luminal fluids, suggesting that it plays a role in epididymal maturation. Because similar biphasic kinetics are seen for HYAL2 and SPAM1 (Sperm adhesion molecule 1), it is likely that HYAL2 plays a redundant role in the catalysis of megadalton HA to its 20 kDa intermediate during fertilization.**

*acrosome reaction, epididymis, fertilization, sperm maturation, zona pellucida*

## INTRODUCTION

Mammalian hyaluronidases (hyases) consists of a highly conserved family of six enzymes [E.C. 3.2.1.25] that catalyze the breakdown of hyaluronan or hyaluronic acid (HA) in the extracellular matrix (ECM) [1, 2]. The mammalian oocyte is endowed with an abundant supply of ECM that is present in a specialized form called the zona pellucida (ZP) in addition to its existence on the surface of the surrounding cumulus cells and within the perivitelline space. Thus, sperm must penetrate formidable ECM barriers to initiate the crucial cell adhesion events necessary for fertilization. They are thought to perform this task with the help of membrane-bound neutral-active hyases that are distinct from three acid-active hyases encoded by genes on a separate linkage group [3, 4], although members of both groups are abundantly expressed in the testis [4, 5]. Neutral-active sperm hyases are multifunctional and are thought to perform roles in addition to the penetration of the HA-enriched vestments. These roles include sperm-ZP binding [6–8] and induction of the acrosome reaction (AR) [9–11].

Sperm adhesion molecule 1 (SPAM1) is the best characterized sperm hyase [3, 7, 12]. In humans and a variety of domestic animals, it has been shown to function bimodally with both neutral and acidic activity [3, 8]. Glycosyl phosphatidylinositol-(GPI)-linked SPAM1 and other sperm hyases are membrane-anchored proteins whose anchors can be cleaved to produce soluble isoforms [9, 13]. They are also expressed in the epididymis where they are secreted and acquired on the sperm surface during epididymal maturation [3, 6, 14, 15]. In humans SPAM1 is the only functional neutral-active sperm hyase, unlike mice where its encoding gene lies within a cluster of three functional family members (*Hyalp1*, *Spam1*, and *Hyal5*) on chromosome 6A2 [3, 4, 16]. With three neutral-active sperm hyases, mice appear to show functional redundancy because deletion of either of the two major genes (*Spam1* and *Hyal5*) does not impair fertility [17, 18]. Interestingly, although in several species SPAM1 was shown to play a role in sperm-ZP binding [6–8, 19], a similar role for both murine SPAM1 and HYAL5 (unique to rodents) has been questioned and the existence of undiscovered sperm hyase(s) was proposed [18].

More recently, we showed that HYAL3, an acid-active hyase encoded by a gene in the cluster of three (*HYAL2*/*Hyal2*, *HYAL1*/*Hyal1*, *HYAL3*/*Hyal3*) on human 3p21.3/mouse 9F1 [4], is expressed and functionally active in sperm [20]. Interestingly, in that study, zymography of murine sperm proteins showed that in the protein fraction from the inner acrosomal membrane (IAM) there was a second hyase at ~57 kDa at pH 4 and that at pH 3 there was a dimer band [20]. These observations raise the possibility that HYAL2, the ubiquitously expressed acid-active somatic hyase with a molecular weight (MW) of ~57 kDa may be present in sperm.

Thus, based on the above observations, we hypothesized that HYAL2 is a germ cell hyase that plays a role is sperm maturation and function. We attempted to test our hypothesis using human sperm samples and murine sperm and epididymal tissues. Using multiple techniques, our results show the presence of HYAL2 in human and mouse sperm and in mouse epididymal tissues and fluids, suggesting that it plays a role in both sperm function and maturation. Its presence in three subcellular compartments where hyase activity is required is consistent with it being a sperm hyase with multifunctional roles. Importantly, we show that immunoprecipitated murine

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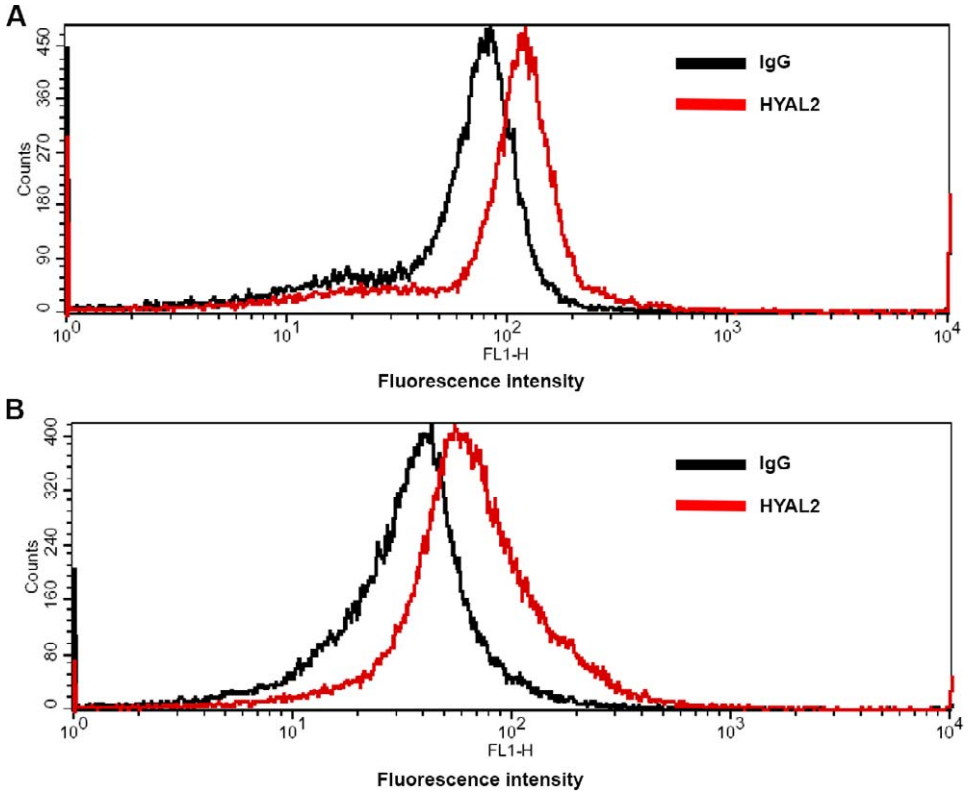


FIG. 1. Flow cytometric analysis of mouse (**A**) and human (**B**) sperm shows HYAL2-specific antibody staining (red) compared to control IgG (black), indicating the presence of HYAL2 on the sperm surface. FITC-conjugated secondary antibodies were used.

sperm HYAL2 is enzymatically active and intimately associated with its cofactor, CD44, which is an HA receptor that is required for its activity in somatic cells [21, 22].

## MATERIALS AND METHODS

### *Animals and Reagents*

Sperm, epididymal and testicular tissue samples, and epididymal fluids were obtained from sexually mature (>3-mo-old) outbred FVBN and inbred C57BL/6 mice as previously described [23, 24]. Studies were approved by the Institutional Animal Care and Use Committee at the University of Delaware and were in agreement with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (publication 85-23, revised 2011). The use of human sperm was approved by the University of Delaware Human Subject Review Board, and informed consent was obtained from the subjects studied. All the enzymes and chemicals were purchased from Fisher Scientific Co., Sigma, or Invitrogen unless otherwise specified.

### *Antibodies*

Affinity-purified rabbit polyclonal human HYAL2 antibodies (ab68608 and ab66653) that cross-react with mouse were obtained from Abcam. The immunogens used in generating the antibodies are unique sequences within residues 1–100 and 350–450, respectively, of the human HYAL2. A rat anti-mouse CD44 monoclonal antibody was obtained from eBioscience, while a human anti-heat shock cognate protein 70 (HSC70) (sc7298) that cross-reacts with the mouse was purchased from Santa Cruz Biotechnology Inc.

### *Localization of HYAL2 and CD44 on Sperm Membrane Using Indirect Immunofluorescence*

*Human sperm samples*. With informed consent, samples were obtained from the Reproductive Associates of Delaware. Fresh liquefied semen or frozen samples in human tubal fluid (InVitroCare) were obtained after standard analysis to assess semen parameters, including sperm concentrations, motility, and morphology, according to WHO guidelines [25]. Sperm were recovered by centrifugation (500 × *g* for 15 min).

*Mouse sperm samples*. The recovery procedure of caudal mouse sperm and the immunofluorescence (IF) protocol for both human and mouse sperm were as described by our laboratory. [20, 24]. Briefly, freshly recovered caudal mouse sperm from two to three males per experiment and sperm from individual males were washed in PBS and fixed with 1.5% paraformaldehyde at room temperature for 1 h. In the case of CD44 localization, cells were permeabilized with 0.1% Triton X-100 in PBS after fixation. They were then washed twice with PBS, blocked for 30 min in 3% bovine serum albumin (BSA) in PBS, and then incubated in the primary rabbit polyclonal anti-HYAL2 antibody (1:300), or mouse monoclonal anti-CD44 antibody (1:200) or both, and the respective rabbit and rat immunoglobulin G (IgG) control. The secondary antibodies for the anti-HYAL2 were Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:600; Molecular Probes) or rhodamine-conjugated anti-rabbit (1:600), while for anti-CD44, it was fluorescein isothiocyanate (FITC)-conjugated anti-rat (1:250). Following incubation with the secondary antibodies, sperm pellets were washed with PBS. For flow cytometric analysis, samples treated with the FITC-conjugated secondary antibodies were resuspended in 1–1.5 ml PBS and analyzed using a FACSCalibur unit (Becton Dickinson) equipped with an argon laser at 488 nm excitation. For microscopic analysis, slides were made from a suspension of the pellets and then counterstained with 4′,6-diaminophenyl-indole (DAPI) in ProLong Gold.

To distinguish between the presence of HYAL2 on the plasma membrane over the head and that on the IAM, immunostaining was performed on acrosome-unreacted and -reacted sperm. AR was performed as previously described after exposure to 20 μM A23187, a $Ca^{2+}$ ionophore, at 37°C for 1 h [12]. Following the PBS washes after treatment with the secondary antibodies, the sperm suspension was incubated with 10 μg/ml of FITC-conjugated peanut agglutinin (PNA) lectin (L7381, donated by Dr. Gail A. Cornwall, Texas Tech University, Lubbock, Texas) and incubated in the dark for 30 min. The cells were then washed in PBS, and slides were made with the suspension and counterstained with DAPI in ProLong Gold. Slides were examined and imaged under a confocal microscope.

### *Preparation of Protein Extracts from Tissues and Sperm for Western Blot Analysis*

Protein extracts were prepared from murine sperm, testes, caput, corpus, and caudal epididymal tissues. Epididymal tissues were washed to remove

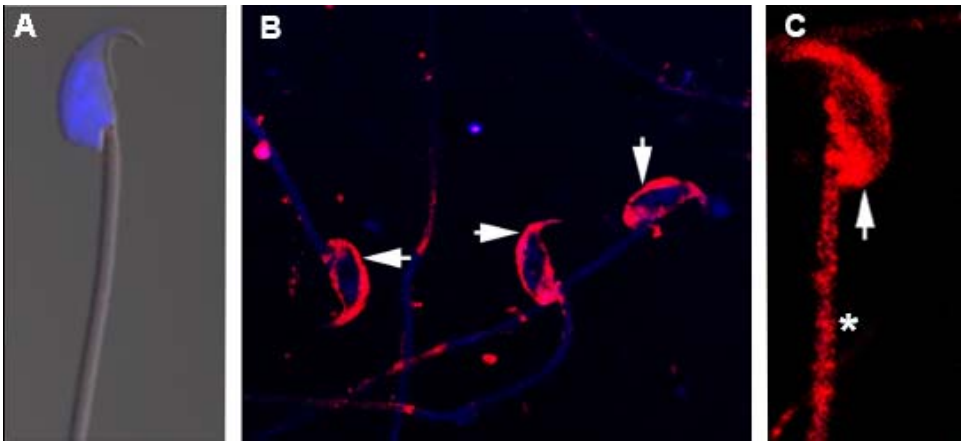


FIG. 2. Indirect immunofluorescence (IF) localizes HYAL2 (red signal) in murine sperm on the plasma membrane over the acrosome (arrowed; **B**), the posterior head (arrowed; **C**), the midpiece, and the proximal principal piece of the flagellum (asterisk; **C**). Control sperm (**A**) treated with rabbit IgG and the secondary antibodies used for sperm treated with the anti-HYAL2 primary antibody shows no fluorescent signal in the merged image stained blue with DAPI. Original magnification ×1000 (**A**–**C**).

sperm and processed as previously described [14, 26, 27]. Epididymal fluids as well as caput/corpus and caudal sperm were recovered as previously described [23, 24]. Sperm samples from five men with motility rates of >40% were used for the protein extracts. Tissues and cells were manually homogenized (using a Dounce homogenizer) in a solubilization buffer (62.5 mM Tris-HCl, 10% glycerol, 1% SDS) at pH 6.8 with Protease Inhibitor Complete (Roche Diagnostics). The suspensions were left overnight at 4°C on a rotor before centrifugation at 14 000 × *g* for 15 min at 4°C to collect the protein-containing supernatant. Protein concentrations were determined, using a bicinchoninic acid (BCA) assay kit (Pierce). Samples were exposed to reducing conditions (99°C for 4 min in the presence of 100 mM dithiothreitol) and processed according to standard protocols [28]. Western blot analysis was performed with the Western Breeze Chemiluminescent Immunodetection Kit according to the manufacturer's protocol. Membranes were probed with the rabbit polyclonal HYAL2 primary antibody (1:500) or anti-CD44 antibody (1:400) overnight and proteins visualized using alkaline phosphatase-conjugated secondary antibodies. Heat shock cognate protein 70 (HSC 70), an abundantly expressed housekeeping protein, was used as a loading control. The membranes were stripped (Restore, Western blot stripping buffer; Thermo Scientific), and the membranes were reprobed with anti-HSC 70 (1:1000). Protein bands were analyzed by densitometry using Image J software, and their ratios to those of the loading control were determined and plotted.

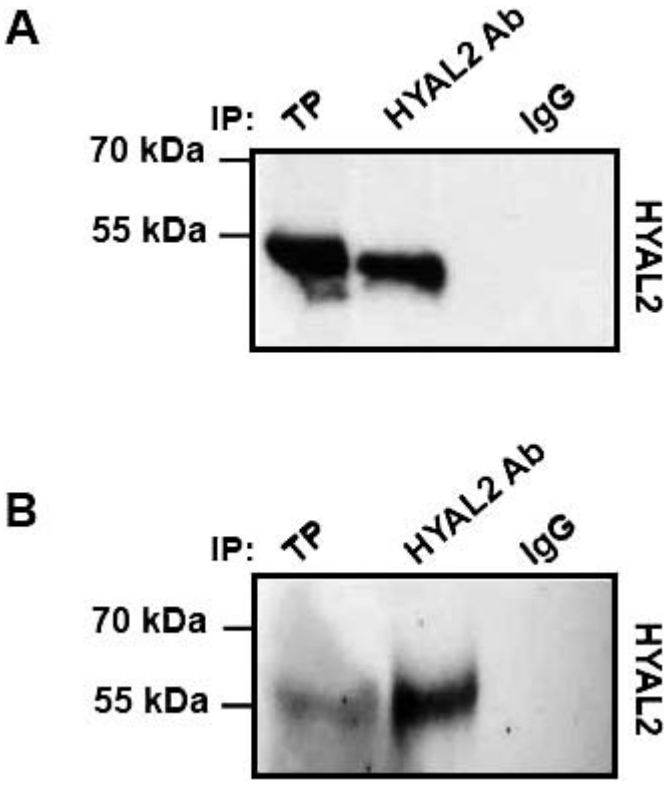


FIG. 3. Immunoprecipitation (IP) assays reveal that the HYAL2 (~57 kDa) is present in mouse (**A**) and human (**B**) sperm lysate. Total protein (TP) from acrosome-intact (AI) sperm is seen in the first lane, while in the second and third lanes, TP was treated with anti-HYAL2 antibody and rabbit IgG. A strong HYAL2 band is seen in both mouse and human for the immunoprecipitated protein, but is absent in the IgG control.

### *Immunoprecipitation of HYAL2 and Coimmunoprecipitation of HYAL2 and CD44*

Protein G magnetic beads (Millipore) were washed twice with 1 ml PBS plus 0.1% Tween 20 and centrifuged at 3000 × *g* for 3 min. The beads were then resuspended in 100 µl of PBS with anti-HYAL2 antibodies or rabbit IgG (control) at a dilution of 1:50 for 2 h at 4°C and placed on a rotor. After incubation, the beads were recovered by centrifugation at 3000 × g for 5 min, washed twice with PBS, and incubated with 125 µg of sperm protein extract (prepared as described above) in 500 µl of immunoprecipitation (IP) buffer (25 mM Tris and 150 mM NaCl, pH 7.2), along with protease inhibitor. After overnight incubation at 4°C on the rotor, the beads were spun at 3000 × *g* for 5 min and washed twice with PBS. The protein bound to the beads was solubilized by boiling in sample buffer, analyzed by 10% SDS-PAGE under reducing conditions, and electrotransferred to nitrocellulose membranes for Western blot analysis using anti-HYAL2 antibodies. The coimmunoprecipitated CD44 was detected by immunoblotting as per standard protocols with the appropriate alkaline phosphatase-conjugated secondary antibodies. Immunoprecipitated protein for the enzymatic activity assay was collected in a nonreducing sample buffer.

### *Sperm Protein Fractionation*

Murine caudal sperm ($5 \times 10^7$) were collected from 3-to 4-mo-old males in PBS and washed, followed by centrifugation at 500 × *g* for 15 min. Proteins from these acrosome-intact (AI) sperm were extracted with 20 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 1% Triton X-100, and protease inhibitor. After solubilization on ice for 6 h, the sperm suspension was centrifuged at 13 000 × *g* for 15 min and the proteins recovered in the supernatant. Sperm were AR as described above. The sperm suspension was then subjected to centrifugation at 500 × *g* for 15 min to collect AR sperm in the pellet from which proteins were extracted as described above. The supernatant was subjected to ultracentrifugation at 100 000 × *g* for 90 min, and the supernatant provided soluble proteins (SAP), similar to that described previously [20, 29], while the pellet provided membrane fragments released after the AR. The pellet was washed with PBS and the proteins extracted at 4°C for 6 h in PBS with 1% Triton X-100, 1 mM ethylenediaminetetraacetic acid, and protease inhibitor, and centrifuged at 13 000 × *g* for 10 min. The resulting supernatant was the

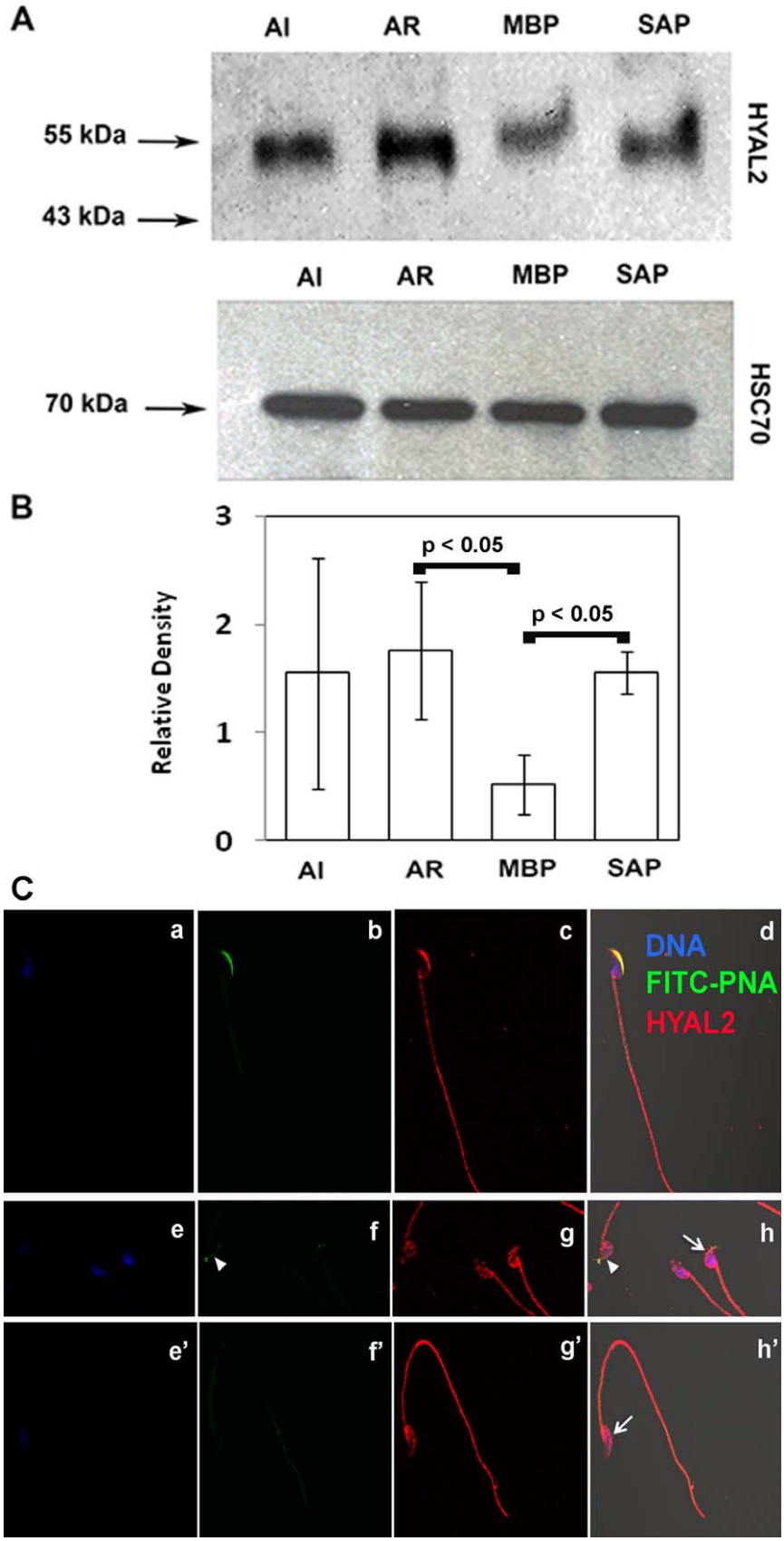


FIG. 4. A broad subcellular distribution of HYAL2 (~57 kDa) is seen in murine sperm, as detected by Western blots of unfractionated and fractionated mouse sperm proteins and by IF. **A**) In this representative blot, a HYAL2 band is seen in unfractionated proteins from acrosome-intact (AI) and acrosome-reacted (AR) sperm as well as the plasma membrane (MBP) and the soluble (SAP) fractions released after the AR (15 μg proteins loaded). In other blots, the SAP fraction appeared to have a slightly smaller MW. The lower panel shows the HSC 70 loading control, after the membrane was stripped and reprobed followed by densitometric analysis of the bands in **B**. The relative densities of the bands indicate that the amount of HYAL2 is significantly ($P < 0.05$)

membranous protein fraction (MBP) released after the AR. The protein concentrations of all the samples were determined with the BCA assay kit.

### Preparation of Total RNAs from Epididymides

Total RNAs from testes, caput, corpus, and cauda epididymides of 3- to 4-mo-old mice were extracted using Tri-Reagent according to the manufacturer's protocol. Prior to extraction of the RNAs, epididymal tissues were minced and washed to remove all the sperm as previously described [14]. RNA samples were subjected to phenol/chloroform extraction and ethanol precipitation.

### Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

First-strand *Hyal2* cDNA synthesis from 2 μg of total RNA was performed with a SuperScript Preamplification System (Qiagen) under the conditions recommended by the manufacturer. Control experiments were performed simultaneously without the addition of RT. Two microliters of each RT product was subjected to PCR amplification using a pair of primers unique for mouse *Hyal2* cDNA: 5′-ACA TAC ACC CGA GGA CTC ACG G-3′ and 5′-TGA ATT CCT TGC ACC AGA GGC CAG-3′ (Integrated DNA Technologies, Inc.). The PCRs were performed under the following conditions: 94°C for 2 min, followed by 34 cycles of 94°C for 1 min, 63°C for 2 min, and 72°C for 2 min, and a final cycle of 72°C for 10 min and holding at 4°C. RT-PCR products were resolved on a 1% agarose gel and stained with ethidium bromide. Four replicate experiments were performed.

### In Vitro Sperm Acquisition of HYAL2 from Epididymal Luminal Fluids as Detected by Flow Cytometry

Epididymal luminal fluids (ELFs) from all three regions were collected, combined, and clarified by centrifugation as described [23, 30, 31]. Protein concentrations were obtained using the BCA kit. Caudal sperm ($2.5–5.0 \times 10^5$) were collected from C57BL/6 mice, incubated at 37°C in clarified ELF with a protein concentration of 1–1.5 mg/ml or in 2% BSA in PBS (negative control) as previously described [30–32]. After incubation, sperm were recovered by centrifugation, washed, and subjected to immunodetection using anti-HYAL2 antibody and the FITC-labeled secondary antibodies. Flow cytometric analysis was used to quantify the amount of HYAL2 on the sperm surface as described above and as previously performed [31, 32].

### HA Substrate Gel Electrophoresis

Hyase activity in sperm proteins was assayed using HA substrate gel electrophoresis (HASGE) as previously described [14, 26, 27, 33]. HA (0.15 mg/ml) from bovine vitreous humor or the 600 kDa polymer (Genzyme) was added to 10% SDS-polyacrylamide gels loaded with 5 or 10 μg of nonreduced proteins, or 25 μl of the 50 μl eluate of IP HYAL2, and run at 15 mA. After electrophoresis, the gels were incubated in 3% Triton X-100 in PBS for 2 h at room temperature, then at 37°C for 36 h in sodium acetate, 25 or 100 mM (pH 4 or 7, respectively). To visualize digestion of HA, gels were stained with 0.5% Alcian blue in 3% acetic acid for 2 h and destained in 7% acetic acid until digestion was visible. Gels were counterstained with 0.25% Coomassie Brilliant Blue G-250 and destained with methanol-acetic acid. Three or more trials were performed at each pH, and the gels were scanned.

### Statistical Analysis

All the experiments were performed at least three times, and the data were analyzed using Image J software. The Student *t*-test and one-way analysis of variance were used to determine if differences in the means ± SEM were statistically significant ($P < 0.05$).

## RESULTS

### HYAL2 Is Present on the Surface of Human and Mouse Sperm Where It Appears in Multiple Compartments

Immunofluorescence, quantified by flow cytometry, revealed the presence of HYAL2 on the membrane of sperm from both humans and mice (Fig. 1). With 50 000 cells analyzed by both the IgG control and HYAL2-specific antibodies, there was a right peak shift for the latter, revealing a positive signal for both species. To confirm the presence of HYAL2 and localize it on the sperm membrane, immunostained murine cells were analyzed by confocal microscopy. HYAL2 was detected over the acrosome and on the posterior head of some cells (Fig. 2B) while in others it was seen on the midpiece and proximal principal piece of the flagellum as well as the other locations (Fig. 2C). In some cases the immunostaining appeared to be punctate (Fig. 2C), suggesting movement of the protein on the sperm surface.

To further confirm the flow cytometric data for the presence of HYAL2 in human and mouse sperm, IP studies were performed on total protein (TP). Figure 3 shows a strong ~57 kDa band, indicating that HYAL2 could be immunoprecipitated from the lysate of mouse (Fig. 3A) and human (Fig. 3B) sperm. While the TP showed a positive band, the IgG control was negative; indicating the specificity of the IP.

We next investigated the subcellular distribution of HYAL2 in murine sperm by performing Western blot analysis of total sperm proteins and protein fractions collected after induction of the AR via $Ca^{2+}$ ionophore treatment. Western blots revealed that the ~57 kDa HYAL2 that is present in the TP resides in all three fractions, namely, AR, MBP, and SAP fractions. Densitometric analysis of the bands, using HSC 70 loading control indicated significantly higher amounts of HYAL2 in the AR ($P = 0.036$) and SAP ($P = 0.006$) fractions compared to the MBP fraction (Fig. 4, A and B). The intense red immunostaining on the head of AR sperm compared to the yellow staining resulting from the merged red and green staining in AI cells in Figure 4C is consistent with the presence of HYAL2 on the IAM.

### HYAL2 Is Present in the Epididymis Where It Is Secreted in a Form in which It Can Be Acquired by Sperm

Because neutral-active sperm hyases and acid-active HYAL3 are expressed in the epididymis where sperm mature [3, 15, 20], we investigated the presence of *Hyal2* expression in the epididymis. RT-PCR was performed on RNA extracted from each of the three epididymal regions after extensive washing of the tissues to remove residual sperm. Using the testis as control, we show the presence of *Hyal2* transcript in all three regions of the epididymis (Fig. 5A). The presence of HYAL2 protein in epididymal tissues was analyzed using Western blots. Figure 5B shows that *Hyal2* transcripts detected in the tissues are translated in all epididymal regions, which showed the ~57 kDa HYAL2 protein band. However, HYAL2 is differentially distributed, with the caput having significantly ($P = 0.02$) higher amounts than the corpus and the cauda where it was lowest. Importantly, HYAL2 is also released in the luminal fluids from all three regions (Fig. 5B). Opposite to what is seen in the tissues, the ELFs had HYAL2 amounts that

greater in the AR and the SAP fractions compared to the MBP. **C**) Confocal images of murine AI (**a**–**d**) and AR (**e**–**h**) sperm show the absence of the FITC-PNA (green) staining in the latter. In the AI sperm, HYAL2 (red) is present on the plasma membrane over the acrosome and when merged with the green appears yellow (**d**). HYAL2 on the inner acrosomal membrane (IAM) appears red in AR sperm in the merged image in the total (arrowed) or near total (arrowhead) absence of the green acrosomal matrix (**h**, **h**′). IgG control without the red signal is not shown. Original magnification ×630.

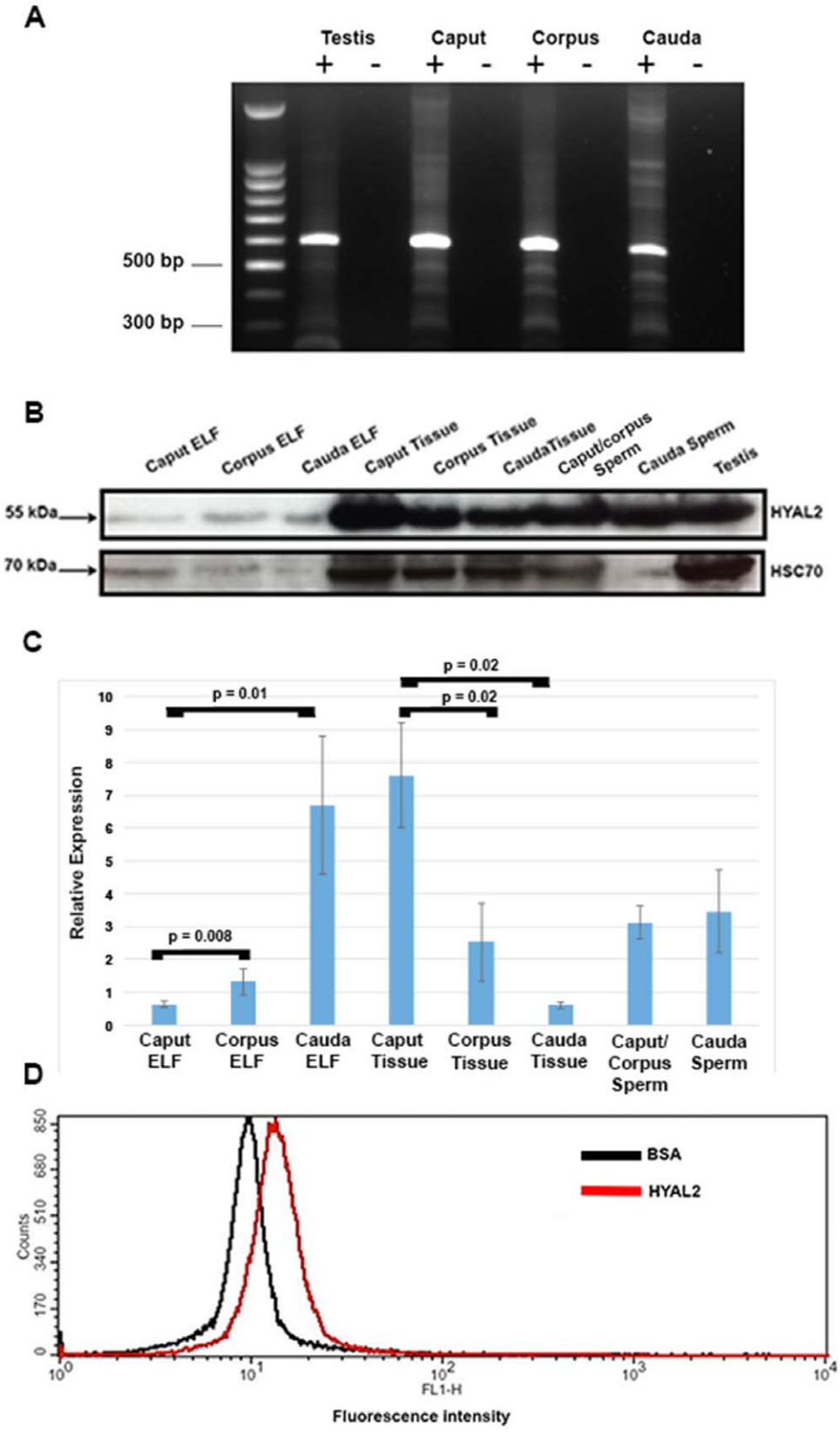


FIG. 5. HYAL2 is present in the murine epididymis, secreted in the ELFs, and can be taken up by sperm coincubated with ELFs. **A**) Steady-state *Hyal2* mRNA, as detected by RT-PCR of the total RNAs from the caput, corpus, and cauda of mouse epididymis. RNA (2 μl) was subjected to first strand synthesis with (+) or without (–) reverse transcriptase, followed by PCR amplification. Products of the expected size are observed for all RNAs and testis (positive control); the 100 bp ladder (M) is on the left. Four replicate experiments gave the identical results. **B**) Western blot analysis reveal the presence of HYAL2 in all three epididymal regions, the corresponding purified ELFs, and caudal and caput/corpus sperm. Testis was used as the positive control, and 40 μg of proteins were loaded in each lane. Blots were stripped and reprobed for HSC70, a housekeeping protein present in ELFs, as a loading control and the bands shown in the lower panel. **C)** The relative expression of HYAL2, as assessed by densitometry of the bands (using Image J software), is shown in the graph. Values are expressed as means ± SD (n = 3), with expression in ELFs highest in the caudal region. Caudal and corpus ELFs had significantly ($P =$

increased from the caput to the cauda. The ~3- and ~8-fold increases in the amounts in the corpus and cauda, compared to that of the caput, are significant ($P = 0.008$ and $P = 0.01$, respectively). When caput and corpus sperm were pooled, they had lower levels of HYAL2 compared to that of caudal sperm.

To determine if HYAL2 in the ELFs can be transferred to the sperm surface in vitro, uptake assays were performed by coincubating mouse caudal sperm and the combined ELFs. Using sperm incubation in BSA as a control and flow cytometric analysis to quantify HYAL2 on the sperm surface, we detected a 4-fold increase in fluorescence intensity in the ELF samples (Fig. 5C). This indicates that HYAL2 was acquired from the ELF during the incubation period, suggesting that ELF HYAL2 is delivered to the sperm surface in vivo.

### *HYAL2 Contributes to the Overall Hyase Activity in Human and Mouse Sperm and Is Enzymatically Active in Immunoprecipitated HYAL2 Where It Is Associated with CD44*

HASGE was used to detect hyase activity at neutral and acidic pH in sperm lysates. Because of the similarity in MWs of the hyases, the activity bands comigrated. Figure 6A shows hyase activity for both mouse and human lysates from three individuals at pH 4 and 7. The comigrated hyase bands ranged from ~44 to 57 kDa in the mouse, while for the human samples, the range was from ~62 to 68 kDa as expected on the basis of the MWs of the hyases in the species [3]. In general, hyase activity bands appeared to be more intense at pH 7 than at pH 4. However, in the human samples, the comigrated bands had a broader MW range at pH 4 than at pH 7.

When mouse sperm proteins were fractionated and hyase activity analyzed at pH 4 and 7, zymography detected activity at both pHs in all three fractions: the AR, MBP, and the SAP (Fig. 6B). The amount of hyase activity varied for the different fractions, being lowest in the SAP fraction and highest in the AR fraction at both pH 4 and 7. For the AR, this pattern is parallel to the distribution of HYAL2 (Fig. 4A). At both pHs, the SAP fraction appeared to have a slightly lower MW, which reflects cleavage of the GPI anchor of the hyases for their release after the AR. Finally, in Figure 6C we show hyase activity of immunoprecipitated HYAL2, using anti-HYAL2 antibodies (ab66653) and for the TP (positive control), but none for the IgG negative control. Activity was also not detected (data not shown) when HYAL2 was immunoprecipitated with the other anti-HYAL2 antibody (ab68608).

To determine if the catalytic activity of HYAL2 is CD44 dependent, as it is in somatic cells [21, 22], we first investigated the presence of CD44 in murine sperm. In Figure 7A we show that CD44 is present on the head, the midpiece, and the proximal principal piece of the flagellum in some cells. Next, we demonstrated that CD44 colocalized with HYAL2 on both the head and the flagellum (Fig. 7B). Importantly, the coimmunoprecipitation assays revealed that HYAL2 antibodies are able to precipitate a very strong CD44 band (Fig. 7C), suggesting that the two proteins interact in sperm.

## DISCUSSION

### *Characterization of the Distribution of HYAL2 in Mammalian Sperm*

While sperm hyases are considered to be neutral-active and encoded by *SPAM1*/*Spam1* and other genes (*Hyal5, Hyalp1*) in the linkage group on human 7q31/mouse 6A2 [1, 4, 16, 34], we have previously shown that *HYAL3*/*Hyal3* in the acid-active linkage group on human 3p21/mouse 9F1 is also expressed in sperm from humans and mice where it is functionally active [20]. Indeed five of the six mammalian hyases, (including two of the three acid-active hyases, HYAL2 and HYAL3) are expressed in the testis [4, 5, 34]. In the present study, we show definitively, for the first time, that the well-characterized acid-active HYAL2, widely distributed in somatic cells [35], is present in human and mouse sperm where it was detected on the plasma membrane by flow cytometric analysis. This finding, which is consistent with it being a well-characterized GPI-linked membrane protein (36), was corroborated by IP assays that revealed the ~57 kDa protein in both species. Thus, our observation is consistent with our earlier finding of a ~57 kDa acid-active hyase in the AR fraction of murine sperm [20] and argues that HYAL2 is a mammalian germ cell hyase.

The IF data also provided insights into the functional roles of HYAL2 in sperm. In the mouse, HYAL2 is present on the plasma membrane over the acrosome, on the posterior head, the midpiece, and the proximal principal piece. These are locations that are typical of SPAM1 and the other sperm hyases [3]. They suggest that HYAL2 may be involved in $Ca^{2+}$ signaling associated with the AR [9–11] as well as with the penetration of the ECM in the cumulus oocyte complex [7, 36]. Similar to the distributions reported for HYAL3 [3, 20], HYAL2 was shown to be most abundant on the IAM of AR sperm and to be present in relatively high levels in the SAP released after the AR. Both of these microenvironments are known to require the presence of the acidic hyaluronidase domain of SPAM1 [8, 19]. Although HYAL2 is least abundant in the plasma membrane fraction, its regionalization on the membrane is interesting. It is found over the acrosome and on the proximal principal piece of the flagellum, both of which are locations of lipid raft microdomains [37] where HYAL2 is known to reside in somatic cells [38]. Thus, it is likely that the functions performed by HYAL2 in sperm occur in a similar microenvironment as those in somatic cells.

The presence of HYAL2 in the soluble acrosomal protein fraction suggests that, like SPAM1 [3, 8], HYAL5 [29], and HYAL3 [20], it is cleaved at the C-terminus near the GPI anchor for its release during the AR. In support of this notion is the finding in Figure 6B that shows that the MW of the soluble hyases appears slightly lower than in the other fractions. As the soluble acrosomal proteins and the IAM, where HYAL2 is most abundant, are both microenvironments requiring the action of acidic hyases for the penetration of the ZP after secondary binding of the sperm [8, 19], our findings suggest that HYAL2 participates in these activities. It is well-established that the acidic hyaluronidase domain of SPAM1 is engaged in the penetration of the ZP [3, 8]. This runs parallel to what occurs in somatic cells where acidic microenvironments are created on lipid rafts where HYAL2 is associated

0.01 and $P = 0.008$, respectively) greater levels of HYAL2 compared to caput ELF, although the levels in caput tissue was significantly higher than those in the corpus and the cauda ($P = 0.02$). **D**) Quantitative IF, detected by flow cytometric analysis, demonstrates the acquisition of HYAL2 by caudal sperm from purified ELFs, following coincubation with combined ELFs. In the 50 000 cells that were examined, there is a 4-fold increase in the fluorescence intensity (peak shift toward the right) of sperm in ELFs compared to the control BSA.

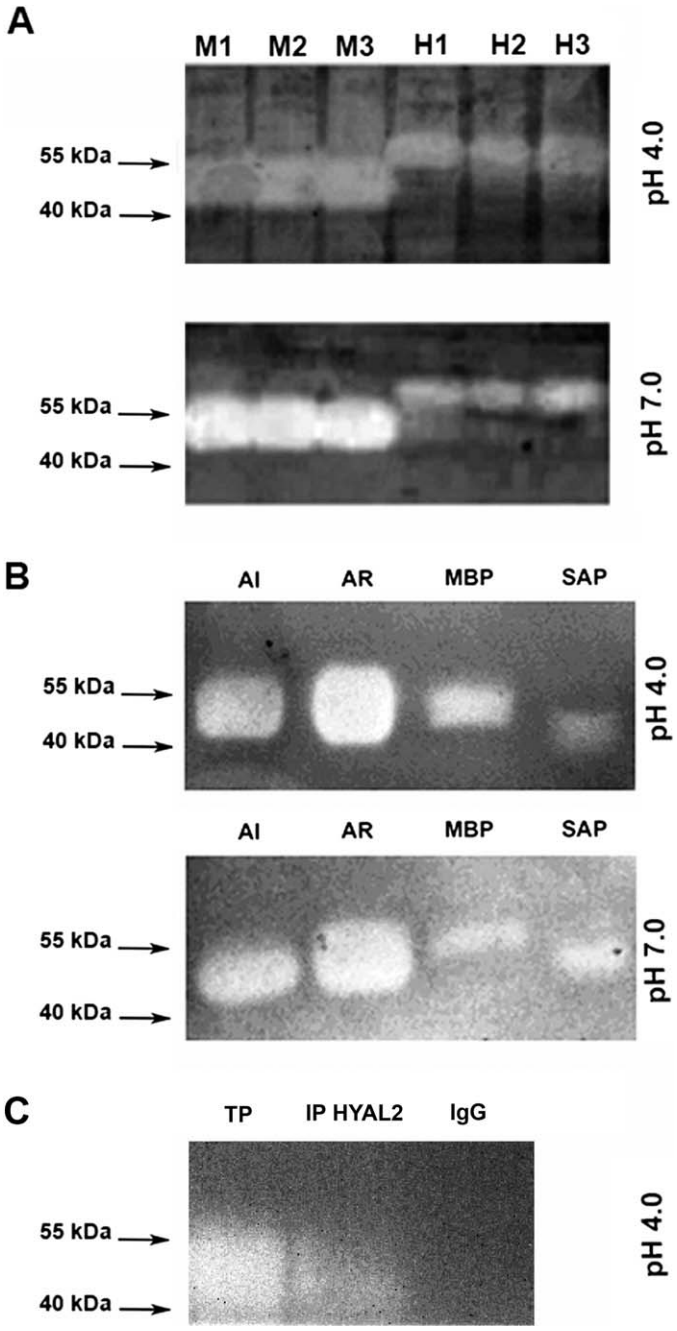


FIG. 6. Human (H) and murine (M) sperm have hyase activity at pH 4 and 7, with zymography showing activity bands to be more intense for the latter. **A**) HASGE analysis, using 10 μg of total protein (TP), was performed on three individuals for each species. The bands from individual hyases comigrate, and those for human samples are seen at MWs ranging from ~62–68 kDa and for the mouse at ~44–57 kDa. The broader bands seen for human proteins at pH 4 than at pH 7 suggest the contribution of multiple acid-active hyases compared to neutral active band(s) (narrower). **B**) HASGE assays of mouse fractionated proteins reveal that neutral and acidic hyase activities are present in all the subcellular locations. The band for proteins from the acrosome-reacted (AR) sperm is the most intense at pH 4 compared to the acrosome-intact (AI) and the membrane-bound (MBP) and soluble (SAP) fractions. It is the broadest, ranging from ~44 to ~57 kDa, consistent with contributions from HYAL3 (MW ~44–47 kDa) and HYAL2. The SAP fractions at both pH 4 and 7 have a lower MW, reflecting cleavage of the GPI anchors. **C**) Immunoprecipitated HYAL2 shows hyase activity at pH 4 where a broad band is seen for the TP and the absence of a band for the IgG control.

with CD44 and the proton pump $Na^+$ $H^+$ exchanger-1 (NHE1) [22].

Because membrane-bound neutral hyase activity is required for the penetration of the ECM [7, 36], the presence of GPI-linked HYAL2 in the sperm plasma membrane fraction suggests that it participates in this functional activity, although its optimal activity in somatic cells is seen at pH 4 [39]. It should be noted that HYAL3, which belongs to the acid-active hyases in somatic cells and is a close family member of HYAL2, was also shown to contribute to the neutral-active hyase activity in sperm and to facilitate the penetration of the cumulus [20]. Thus, HYAL2, like SPAM1 and HYAL3, may have bimodal activity in sperm and its presence on the plasma membrane on both the head and the flagellum is consistent with a contributing role in the penetration of the cumulus oocyte complex.

### *Epididymal Expression of HYAL2 and Its In Vitro Acquisition on Caudal Murine Sperm*

This study reveals that HYAL2 is epididymally expressed. Both *Hyal2* mRNA and protein were detected in all three regions of the epididymis, a finding similar to that reported for human and mouse sperm hyases [3, 15, 26]. Interestingly, the quantitative distribution of epididymal HYAL2, with significantly decreasing amounts from the proximal to the distal (caput to cauda) regions, appears to be different from that of epididymal SPAM1 where the protein level is highest in corpus epididymis [14]. The amounts of HYAL2 in the ELF from the three regions did not mirror those in the tissues because they increased significantly from caput to cauda. This suggests that although the caput and corpus tissues have more HYAL2 than caudal tissue, they are likely to have a smaller proportion distributed on the apical surface of the epididymal epithelium from where epididymal proteins are released in epididymosomes via apocrine secretion [40].Therefore in the caput and corpus a greater proportion of the HYAL2 may be involved in the maintenance of the tissues compared to that secreted from the epididymal epithelia.

Because sperm are transcriptionally inactive, the greater amount of HYAL2 in caudal sperm, compared to that in caput/corpus, may be explained by uptake of HYAL2 from ELF. Although the increase was not significant, the higher level of HYAL2 secretion in caudal ELF is consistent with its higher level in caudal sperm, and the difference in sperm HYAL2 might have been more pronounced if a comparison had been made between those from the caput and cauda. With the highest level of ELF HYAL2 present in the cauda, it is expected that sperm HYAL2 amounts would increase the longer they are stored. Therefore, the expression pattern of HYAL2 in ELFs suggests that HYAL2 is involved in both the epididymal maturation and storage of sperm [41 ,42], a finding similar to that reported for SPAM1[15].

It should be noted that the soluble HYAL2 released in the ELFs is different from the soluble HYAL2 fraction released from sperm during the AR. As mentioned above, in the latter the GPI anchor is likely cleaved, while in GPI-linked proteins released from the epididymal epithelia in the ELFs the anchor is intact [23, 32]. These GPI-linked epididymal proteins have been shown to be associated with lipid carriers [32] or with membrane vesicles called epididymosomes [31, 40] from which they are delivered to the sperm surface [31, 32, 43]. The data from the in vitro uptake assay revealed a 4-fold increase in HYAL2, after coincubation of caudal sperm and ELFs (Fig. 5D). This observation suggests that HYAL2, shown here to be a secretory protein, is acquired by sperm during their

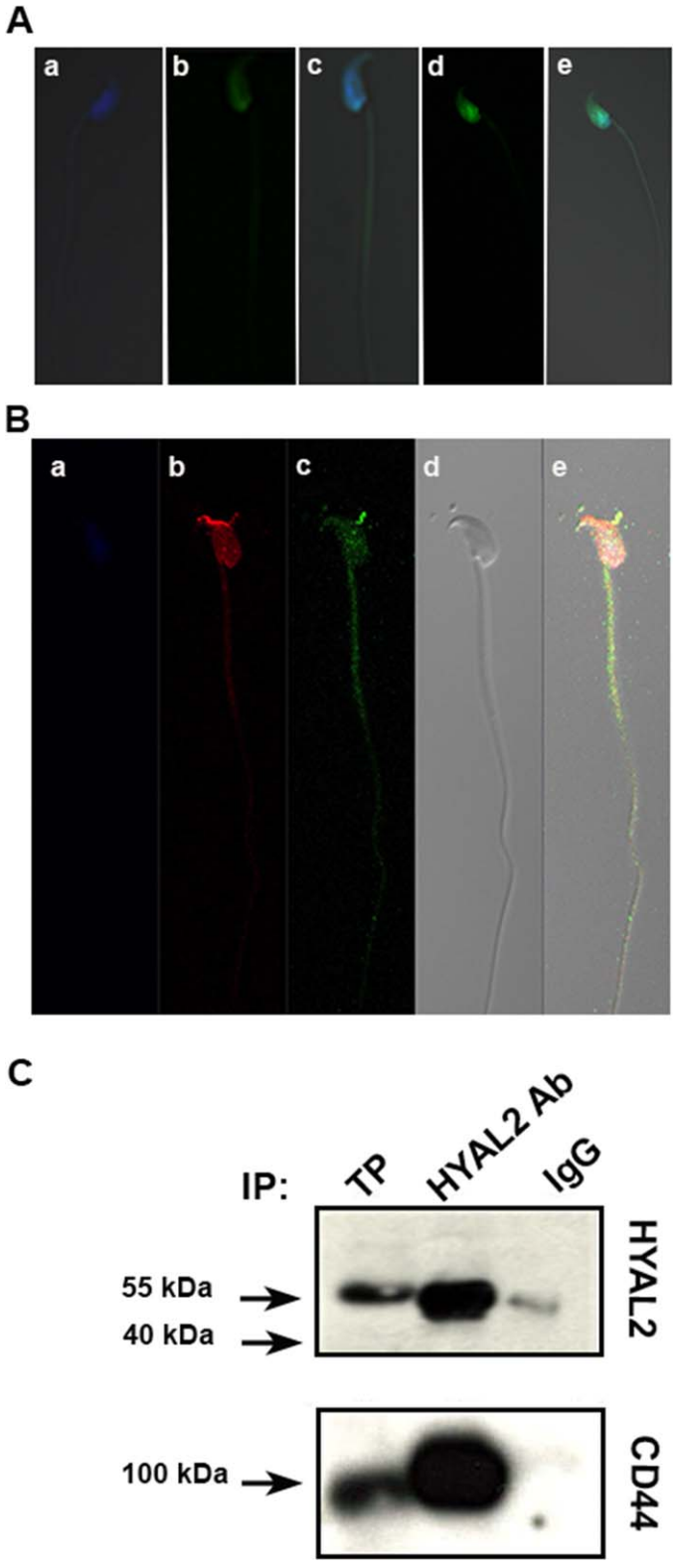


FIG. 7. CD44 is present in murine sperm where it colocalizes and coimmunoprecipitates with HYAL2. **A**) Immunofluorescence shows the presence of CD44 (green) evenly over the head (**b**–**e**) and the midpiece and the proximal principal piece (**b**, **c**) compared with control sperm (**a**) treated with IgG and the secondary antibody conjugated with Alexa Fluor 488. Sperm were counterstained with DAPI (blue), and merged images of **b** and **d** are seen in **c** and **e**. Original magnification ×630. **B**) Colocalization of (**b**) HYAL2 (red) and (**c**) CD44 (green) with the merged image seen in **e**. Control sperm treated with the IgGs is seen in **a** while **d** shows the differential interference contrast imaged sperm. Original magnification ×630. **C**) Coimmunoprecipitation of murine HYAL2 and CD44, using HYAL2 antibody to precipitate HYAL2 (upper panel). After stripping the blot and reprobing with anti-CD44, a strong ~100 kDa CD44 protein was detected (lower panel) for the IP and the TP.

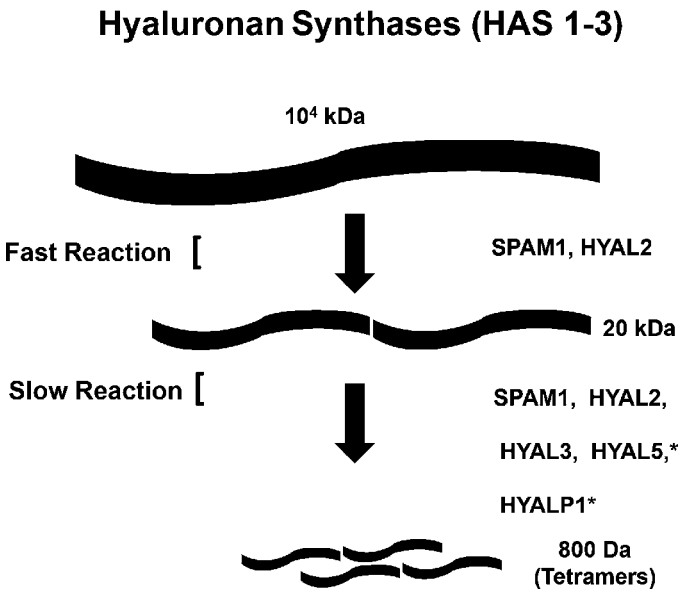


FIG. 8. Schematic representation depicts the proposed catabolism of hyaluronan (HA) in mammalian sperm. Hyaluronan chains up to 10 000 kDa are made by HA synthases (HAS 1–3). Only SPAM1 and HYAL2 are capable of using the large megadalton HA chains as a substrate [1, 48, 49]. In the first step, a reaction with rapid kinetics, they degrade megadalton HA to 20 kDa fragments [48, 49]. In a second step in which the reaction kinetics is slow, they catabolize the 20 kDa fragments to 800 Da tetramers [48, 49]. HYAL3 and neutral-active hyases unique to rodents (HYAL5* and HYALP1*) contribute to the degradation of 20 kDa fragments to 800 Da tetramers [3, 18, 20].

epididymal transit and storage, as part of their maturational process. This conclusion is consistent with increased amounts of HYAL2 on caudal sperm compared to that seen in immature epididymal sperm (Fig. 5C).

### *Enzymatic Activity of HYAL2 in Sperm*

In both humans and mice, HASGE assays detected hyase activity in total sperm proteins at pH 4 and 7, with the bands being more intense at pH 7. However, at this pH the bands for human sperm were narrower (Fig. 6A), reflecting the contribution of the sole neutral-active hyase, SPAM1, compared to three such hyases in mice [3]. Further, in addition to SPAM1, which is bimodal with a broad pH activity range (3.6–8.6) in a variety of species [8, 44, 45], acid-active HYAL3 is known to exist in human and mouse sperm [20]. Thus, in the present study, the detection of HYAL2 in both species brings to two the number of acid-active sperm hyases, in addition to SPAM1 with its bimodal activity, and is consistent with a broader activity band in humans at pH 4 than at 7. However, the intensity of the activity at pH 4 is weaker, consistent with the weak activity reported in somatic cells for HYAL2 [39] and HYAL3 [46].

For murine sperm, the results also corroborate our earlier finding that there is hyase activity at pH 4 [20]. This is in contradiction to another report that showed the absence of activity below pH 5 [29]. When the presence of HYAL2 was analyzed in fractionated sperm proteins, we detected its highest

distribution in the AR fraction (Fig. 4A) where the highest level of hyase activity was detected at pH 4 (Fig. 6B). This is consistent with our earlier study in which a ~57 kDa hyase was present in the AR fraction at pH 4 [20] and strongly suggests that HYAL2 contributes to hyase activity at pH 4 on the IAM of AR sperm. Unsurprisingly, HYAL2 is detected in relatively high levels in the SAP fraction, which is known to show acidic hyase activity [8]. The finding of hyase activity in immunoprecipitated HYAL2 conclusively reveals that HYAL2 is enzymatically active in murine sperm at pH 4. Our inability to detect activity when the immunogen used for the antibody is within residues 1–100, but not within 350–450, likely reflects antibody blockage of the catalytic domain of HYAL2, which is close to the amino-terminal end of the protein.

Because HYAL2 hyase activity in somatic cells is CD44 dependent [21, 22], we used IF to localize CD44 in murine sperm and showed that it is present on the head, the midpiece, and the proximal principal piece of the flagellum. While CD44 has been shown to be present in human sperm [47], this is the first report of its presence or localization in mouse sperm. Further, we showed that murine CD44 colocalized and coimmunoprecipitated with HYAL2. The latter indicates that HYAL2 and CD44 are in intimate association, which is a requirement for HYAL2's enzymatic activity [21, 22]. This association of HYAL2 with its CD44 cofactor is consistent with HYAL2's enzymatic activity in sperm. In somatic cells, the association is known to promote acidification of the extracellular environment and to activate HYAL2 [22].

With the identification of HYAL2 in sperm, the only somatic hyase that is not found in germ cells is HYAL1. HYAL1 has the ability to catabolize very large MW (10 000 kDa) HA, which consists of β-1,4-linked disaccharide units of glycosaminoglycans, to 800 Da tetramers [34]. In sperm, neutral-active SPAM1 has been reported to catabolize 20 kDa HA to 800 Da tetramers [1, 2]. Because neutral hyases in sperm use 20 kDa fragments of HA as their substrate, in the absence of HYAL1 in sperm, this substrate must be generated by another hyase.

With the availability of soluble HYAL2 in which the GPI anchor has been removed, more recent studies have shown that HYAL2 is capable of degrading heterogeneous high-MW HA polymers [48, 49]. HYAL1, HYAL2, and SPAM1 have been reported to have biphasic kinetics with a rapid initial catalytic activity that generates a stable 20 kDa intermediate equivalent to 50–60 disaccharides that is more slowly degraded into 800 Da tetramers [48, 49]. Because we have identified soluble HYAL2 in sperm (Fig. 4A) with enzymatic activity that degrades heterogeneous high-MW (9–300 kDa, average 63 kDa) HA polymer of bovine vitreous humor (Fig. 6C), we propose a model for the stepwise catabolism of HA in mammalian sperm (Fig. 8). In this model, both SPAM1 and HYAL2 degrade high-MW HA in a fast reaction to the 20 kDa intermediate, followed by a slow degradation to the tetramers. This slow degradation appears to require the contribution of other hyases: HYAL3, HYAL5, and HYALP1. Thus, it appears that SPAM1 and HYAL2 have redundant roles in the degradation of HA, a scenario that explains the normal fertility phenotype seen in *Spam1* nulls [17] and the fact that *Hyal2* nulls are fertile [50]. Our model also explains the fertility of *Hyal5* nulls [18] in the presence of *Hyal3, Spam1, Hyalp1,* and *Hyal2*.

However, because sperm hyases are multifunctional proteins and because in somatic cells HYAL2 serves a unique role as a cell-entry receptor for viruses [49], it is possible that HYAL2 may also have a unique nonenzymatic role in sperm. This may help explain the inability to obtain *Hyal2* nulls from matings of $Hyal2^{-/+}$ in the C57Bl/6 inbred strain [51] as well as the distorted segregation ratios obtained even when crossings were made on an outbred background [50]. The distorted segregation ratios observed suggests there may be a disadvantage to *Hyal2*-null sperm. Further studies will be focused on examining sperm function in *Hyal2* nulls generated on an outbred background, which is conducive not only to their survival [50], but also to their production of zygotes. It would be interesting to determine if HYAL2, which is a relatively weak hyase compared to SPAM1 [39], is upregulated in *Spam1*-null sperm and vice versa.

In conclusion, the finding in this study that enzymatically active HYAL2 and its cofactor, CD44, are present in sperm in an acidic microenvironment argues cogently that it is a germ cell hyase and brings the numbers of such hyases to five and three in mouse and human sperm, respectively. HYAL2 shares with SPAM1 biphasic kinetics and appears to have bimodal activity in sperm where its presence may explain the fertility of *Spam1* and *Hyal5* nulls. Functional redundancy of both acidic and neutral active sperm hyases underscores the importance of these proteins, which play essential multifunctional roles in the steps of fertilization.

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