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MOUSE TESTICULAR HYALURONIDASE-LIKE PROTEINS, SPAM1 AND HYAL5 BUT NOT HYALP1 DEGRADE HYALURONAN

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Running title: Characterization of mouse sperm hyaluronidases

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Abbreviations used: Hyaluronan, HA; extracellular matrix, ECM; glycosylphosphatidylinositol, GPI; glycosaminoglycan, GAG; phospholipase C, PLC

Abstract:

Besides SPAM1 (formerly named PH-20), further hyaluronidase-like proteins, HYAL5 and HYALP1 are also expressed in murine testicular tissue. As they share a high degree of sequence similarity with known hyaluronidases, all three polypeptides could potentially exhibit hyaluronidase activity, a function that is beneficial for sperm in order to penetrate the hyaluronan-rich cumulus, which surrounds the oocyte. Recently, it was reported that SPAM1-deficient mice are fertile and sperm derived from mutant mice still exhibits hyaluronidase activity (Baba et al., (2002) *J. Biol. Chem.* 277, 30310-30314). We have now recombinantly expressed mouse SPAM1, HYAL5 and HYALP1 in *Xenopus laevis* oocytes and determined their respective expression pattern in testis. Transcripts of all three genes are expressed in seminiferous tubules in regions, where maturing spermatogenic cells reside. SPAM1 and HYAL5 but not HYALP1 proteins exhibit hyaluronidase activity at neutral pH. The two active hyaluronidases are both bound to the cell surface via a glycosylphosphatidylinositol anchor. Furthermore, structural characteristics are discussed that are necessary for hyaluronidases in order to exhibit hyaluronan cleavage.

Introduction

Hyaluronan (HA), a polymer consisting of repeating β -1,4- linked disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid, is a common glycosaminoglycan present in the extracellular matrix (ECM) of connective tissues (1). HA is implicated in many physiological processes, including cell migration, proliferation, and differentiation. Hyaluronidases, enzymes that hydrolyse HA to oligosaccharides, have been identified in mammalian, insect, and bacterial species (2, 3).

HA accumulates between cumulus cells (4) together with inter- α trypsin inhibitor (5) as well as a variety of other matrix proteins (6-8). The jelly-like shell protects the oocyte and facilitates its extrusion at ovulation (9). Furthermore, the swollen cumulus is more easily captured by the fimbria of the oviduct and thus supports oocyte transportation to the site of fertilization (10). The tight matrix however is difficult to perforate by mechanical means. Sperm hyaluronidase activity positively correlates with fertilization rate, because sperm that possesses high motility and fertilization efficiency penetrates highly viscous solutions of HA at a higher rate (11, 12). Therefore, the motility and sperm hyaluronidase activity are thought to facilitate path finding through the extracellular matrix of cumulus cells, since no proteases have yet been implicated in this process (13). A widely conserved mammalian sperm surface protein, PH-20 (14), also called sperm adhesion molecule 1 (SPAM1) (15, 16) is well known for its hyaluronidase activity (17, 18). This glycosylphosphatidylinositol-anchored (GPI) sperm hyaluronidase has multiple roles in fertilization (19). SPAM1 and the soluble form of HYAL2, sHYAL2 exhibit activity at both neutral and acidic pH (20), whereas for other hyaluronidases, enzymatic activity appears to be restricted to either acidic pH, e.g. HYAL1 (21) or neutral pH as known for *Xenopus* XKH1 (22).

Unexpectedly, mice carrying a null mutation in the *Spam1* gene are fertile (23). *In vitro* fertilization assays showed that mouse sperm, lacking SPAM1, possesses a reduced ability to disperse cumulus cells from the cumulus mass, resulting in delayed fertilization solely at the early stages after insemination. In this particular context, sperm extracts derived from mutant mice contain at least one

further hyaluronidase, HYAL5, which is active in solutions of both neutral pH and extracellular ionic composition (24).

In the mouse genome, there are seven hyaluronidase-like sequences, six of them clustered in groups of three at two chromosomal sites, on chromosomes 9 F1–F2 (*Hyal1*, *Hyal2* and *Hyal3*) and 6 A2 (*Hyal4*, *Spam1/PH-20* and *Hyalp1*) (23, 25, 26). The orthologous human genes are localized on syntenic regions, on chromosomes 3p21.3 and 7q31.3. *HYALP1* is an expressed pseudogene in humans (27). In mouse, *Hyalp1* shows more characteristics of a gene that codes for a transcript that can be translated into a polypeptide chain similar to SPAM1. The seventh mouse hyaluronidase-like gene, tentatively called *Hyal5*, is localized in close proximity to the cluster at chromosomal region 6 A2. So far, no further hyaluronidase-like gene has been discovered in the human genome at this position. Both genes, *Hyalp1* and *Hyal5* encode open reading frames for a polypeptide highly similar to biochemically characterized hyaluronidases (26). Moreover, these three genes are strongly expressed in testis. So far, none of these murine gene products has been recombinantly expressed and assayed for enzymatic activity. Hence, HYAL5, HYALP1 or both could actually be functionally involved for fertilization in mouse. We therefore expressed these proteins in frog oocytes and studied their enzymatic properties with respect to HA degradation.

Material and Methods

Frog surgery, oocytes preparation, cDNA construction, cRNA injection - A small lobe of ovary was surgically removed from an anaesthetized adult *Xenopus laevis* female. The ovarian tissue was rinsed in O-R2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, 5 mM HEPES, pH 7.8) and individual oocytes were defolliculated manually. The coding regions of Spam1, Hyal5, and Hyalp1 were cloned into pT7TS (gift from P.A. Krieg, Univ. of Arizona, Tucson, AZ, USA), suitable for generating *in vitro*-transcribed mRNA. Hyal5 cDNA (clone ID IMAGp998B198849.1) was purchased from the German Resource Center Berlin (RZPD). The protein coding region of Spam1 as well as Hyalp1 was amplified by PCR using cDNA derived from mouse testis as a template, and for Spam1 primers 5'-GGTGATCAATGGGAGAGTTGAGATTTAAGCACCTC-3' and 5'-CCGCTAGCC TAAGGAGTACTGACTAGTGTCTTCCG-3', while for Hyalp1, primers 5'-TCGAGATATCATGTTTATCCAGTGGGTGACACAG-3' and 5'-GGACTAGTTT TAGAAAATGCTTGAAATCAAAGA-3' were used.

About 50 ng of capped cRNA was injected with the aid of a Nanoliter Injector (World Precision Instruments) into oocytes that were then cultured in O-R2 containing 100 U/mL penicillin and 100 µg/mL streptomycin at 16°C for up to 2 days. GPI-linked proteins were removed from the plasma membranes using 50 mU of *Bacillus cereus* phospholipase C (PLC) (Molecular Probes) in 30 µL of O-R2 buffer, pH 7.0 containing 4 mM CaCl₂, 50 µg/mL BSA per oocyte at ambient temperature for 1 hour.

Expression analysis - For expression analysis, RNA was extracted as described previously (28). Mice were dissected. Specimens were carefully rinsed in PBS and snap frozen in liquid nitrogen. Cells or pieces of tissue were homogenized in 4.2 M guanidinium thiocyanate, 100 mM HEPES pH 7.5. The resulting solution was extracted with phenol:chloroform:isoamyl alcohol (25:50:1) and the aqueous phase supplemented with 10 (v/v)% of 2 M sodium acetate, pH 4.4, and 20 (v/v)% water and extracted with phenol:chloroform (3:1). The RNA within the aqueous phase was precipitated by addition of an equal

volume of 2-propanol. The RNA pellet was rinsed with 80% ethanol, dried and dissolved in 6 M urea, 100 mM HEPES pH 7.5. As a final purification step the RNA was precipitated by the addition of 50 (v/v)% 9 M LiCl. 5 µg of total RNA derived from various tissues and organs were separated by 1.2 % agarose gel electrophoresis and subsequently transferred to Zetaprobe nylon membrane (BIORAD). After UV-crosslinking, blots were hybridized with ³³P-labelled cDNA probes. RT-PCR assays were carried out using specific primer pairs for α -actin, Hyal5 and Hyalp1 on template cDNAs derived from a range of C57BL/6J mouse tissues, and from a murine Sertoli cell line, TM4 (29). PCR was carried out in volumes of 25 µL, containing 1 µM primer and 2.5 units Pfu DNA polymerase in reaction buffer with MgSO₄ (as recommended by the manufacturer, Fermentas) for 30 and 40 cycles at 95°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute. Subsequently, the products were analyzed on a 2% agarose gel. Quantitative RT-PCR assays were performed with the aid of a LightCycler instrument and LightCycler 3.5 software (Roche). The reactions were carried out in a volume of 15 µL with the LC-FastStart DNA Master SYBR Green I Kit (Roche), 2 µM primer and 3 mM MgCl₂. After the activation of the enzyme at 95°C for 7 minutes, 45 amplification cycles were performed at 95°C for 15 seconds, 58°C for 8 seconds and 72°C for 12 seconds. The following oligonucleotide primers were used in order to amplify cytoplasmic α -actin: fwd 5'-GGCTGTATTCCCCTCCATCG-3', rev 5'-CCAGTTGGTAACAATGCCATGT-3'; Hyal5: fwd 5'-AAAGACCCTCGAAATCCAC-3', rev 5'-CCACATACCATTCCAGAGG-3'; and Hyalp1: fwd 5'-TGTCACCTTTGTGGAGATCGGA-3', rev 5'-GCCAACGTGAGAGTAACATTCA-3'. These primer pairs yielded amplification products of 154 base pairs (bp) for α -actin, 144 bp for Hyal5 and 222 bp for Hyalp1. The relative abundance of Hyal5 and Hyalp1 transcripts was calculated relative to α -actin, and the values were normalized to the expression level in testis.

In situ hybridization was performed as described previously (30). Briefly, testicular tissues from C57BL/6J mice were fixed in phosphate buffered saline containing 4% *para*-formaldehyde. The tissue was embedded in Paraplast Plus (Sigma), sectioned to a thickness of 7 µm and mounted on poly-L-lysine coated microscope slides. After treatment with proteinaseK and prehybridization, sections were incubated

at 50°C with digoxigenin-labelled RNA probes in hybridization buffer containing 50 (v/v)% formamide, 1xSSC, 1x Denhardt's solution, 5 mM EDTA, 50 µg/mL yeast RNA, 0.2% Tween 20, 0.5% CHAPS, 10 µg/mL heparin. Thereafter, sections were washed with hybridization buffer and the probes were detected using anti-digoxigenin alkaline phosphatase Fab conjugate and BM Purple as a substrate (Roche).

Hyaluronidase activity measurement - Enzyme activity was measured as described previously (31).

Briefly, enzymes were mixed with 5-aminofluorescein-labelled HA and incubated at different pH values (100 mM sodium phosphate buffer containing 50 µg/mL BSA and 0.1% Triton X-100 first adjusted with citric acid to pH 3.5 and then titrated to the appropriate pH with sodium hydroxide). The reaction mixture was separated by 1% agarose gel electrophoresis. The gel was subsequently blotted onto a positively charged nylon membrane (Biodyne PLUS, 0.45 µm, Pall Life Sciences) and the hapten was detected after incubation with anti-fluorescein alkaline phosphatase Fab conjugate (Roche) by means of enhanced chemiluminescence technology (Amersham Biosciences).

Molecular modelling – Tertiary structural models for the hyaluronidase domains of SPAM1, HYAL5 and HYALP1 were generated applying homology modelling of SWISS-MODEL (32) using its alignment interface together with mouse hyaluronidase-like protein sequences of SPAM1 (accession number AAP49832), HYAL5 (accession number BAC55071) and HYALP1 (accession number BAB30316) pre-aligned with the aid of ClustalW and the X-ray structure of bee hyaluronidase (PDB accession code: 1fcv). Images were generated with the aid of DeepView/Swiss-PdbViewer version 3.7 (33).

RESULTS

Structural Features - SPAM1, HYAL5 and HYALP1 were found to share conserved elements with hyaluronidase from honeybee. Besides the first highly conserved N-terminal domain, all mammalian hyaluronidase-like proteins have a second domain at the C-terminal part of the amino acid sequence. For SPAM1 type hyaluronidases, processing and GPI anchor linkage is needed at the carboxy terminus in order to bind the enzyme to the outer surface of the plasma membrane (34). Bioinformatic prediction of whether one of the three different mouse proteins would contain a consensus pattern for GPI linkage, yielded inconclusive results (35, 36). Therefore, it did not become clear from these theoretical considerations, which one of the three hyaluronidase-like genes would potentially code for a HA-degrading enzyme, and thus is functionally involved in fertilization in mouse.

Expression Pattern - Using Northern blots, Hyal5 as well as Hyalp1 were found to be expressed in mouse testis (Fig. 1A). Like Spam1, transcripts of the other two genes are barely detectable within other tissues (Fig. 1B). Applying quantitative reverse-transcription PCR techniques, trace amounts of both Hyal5 and Hyalp1 could be detected in epididymis. This may well be due to sperm present in the highly convoluted epididymal duct. In TM4 Sertoli cell line (29, 37), only Hyal5 transcripts, yet no Hyalp1 transcripts could be detected. A small number of Hyalp1 transcripts as compared to the amount found in testis was also detected in liver, bone marrow, brain, fat, skeletal muscle, lung and spleen as well as in epididymis (Fig. 1B, C). As shown previously, SPAM1 and HYAL5 proteins appear to be present on acrosome intact sperm (23). Furthermore, HYALP1 has been found not only in testis and caudal sperm but also in sperm-free epididymis (24).

The precise distribution of Spam1, Hyal5 and Hyalp1 transcripts in testicular tissue, was determined by in situ hybridization (Fig. 2). All three hyaluronidase-like transcripts show an equivalent expression pattern of a ring-shaped area within seminiferous tubules (Fig. 2B-D). Some of the sectioned tubules show weak to no signals for Spam1, Hyal5 and Hyalp1 expression. For controls, hybridizations with sense probes

were performed in parallel. Low background and no specific signals were observed (Fig. 2B-D, inlaid micrographs). In sub-human species, a particular cellular association consisting of spermatogenic cells of a distinct stage occupies a relatively long segment along the tubule. High expression of Hyal5 or Hyalp1 transcripts in Sertoli cells appears unlikely since only minor amounts were detected in cultured TM4 cells. We therefore deduced from this expression pattern that transcripts of mouse hyaluronidase-like genes are accumulating in spermatogenic cells, most probably in pachytene and/or leptotene spermatocytes (stage I, II, V, and/or VI). No expression was found near or at the basal lamina, where type A and B spermatogonia, preleptotene spermatocytes of stage III and IV reside. Due to the expression pattern, we conclude that all three polypeptides, SPAM1, HYAL5 and/or HYALP1 protein are present in spermatids and could thus also bound to mature sperm.

Enzymatic activity – Since mouse HYAL5, HYALP1 and SPAM1 have not been recombinantly expressed to examine hyaluronidase activity, we translated *in vitro* transcribed cRNA of all three candidates in intact *Xenopus laevis* oocytes. Cell lysates were tested for hyaluronidase activity using a sensitive assay (31). For HYAL5 and SPAM1, hyaluronidase activity was found in a broad pH range at both acid and neutral conditions. Residual activity could still be measured at pH 7.5 (Fig. 3). In contrast to other hyaluronidases such as HYAL2, which primarily generates intermediate length reaction products of around 20 kD in size, both HYAL5 and SPAM1 were found to degrade HA to short fragments. No hydrolysis was detectable after reaction of HYAL5 and SPAM1 expressing oocytes with glycosaminoglycans (GAG) such as chondroitine sulfate A, B, C or heparan sulfate as substrates (data not shown). For recombinant HYALP1, no hydrolyzing activity with respect to HA was detectable though Hyalp1 and Hyal5 cRNA were equally well translated *in vitro* in the presence of rabbit reticulocyte lysate (Fig. 4). When cell-free expression systems together with microsomal preparations were used for the translation of cRNAs that encode well-characterized hyaluronidases, in no case were enzymatically active protein products obtained. Hence, we extensively tested HYALP1 expressing oocytes or corresponding culture supernatants, in order to detect the presence of trace amounts of HA-degrading activity. As positive controls, besides SPAM1 and

HYAL5, human PH-20, HYAL1, honeybee hyaluronidase and *Xenopus* XKH1 (22) as well as XHyal2 (38) were enzymatically active, when expressed in *Xenopus* oocytes. Yet no HA hydrolyzing activity was detectable after Hyalp1 cRNA injection neither in cells nor in the culture supernatant. Furthermore, chondroitine sulfate A and C as well as heparan sulfate were not depolymerized by HYALP1 expressing oocytes (data not shown).

Next, frog oocytes expressing HYALP1, HYAL5 and SPAM1 were incubated with PLC in order to test whether the enzymes had been linked via GPI anchors to the cell surface. In the cases of HYAL5 and SPAM1, enzyme activity was found in the supernatant of PLC-treated oocytes (Fig. 5). HYAL2, a hyaluronidase that is normally bound to the plasma membrane via a GPI anchor and inactive at neutral pH (39), was however found to exhibit weak enzymatic activity also at neutral pH, when recombinantly expressed in its soluble form, sHYAL2 (20). In the case of HYALP1 expressing oocytes, no HA degrading activity was detectable in the supernatant. Notably, the rat homologue of SPAM1, 2B1 glycoprotein (PH20) is also not rendered free from the cell surface by PLC-treatment (40). We therefore have to admit that HYALP1 could be linked to the membrane and only exerts enzymatic activity after being released by a yet unknown process. In conclusion, HYAL5 and SPAM1, after entering the secretory pathway, are sorted and eventually bound through a GPI-anchor to the plasma membrane as active hyaluronidases.

DISCUSSION

In testicular extracts, hyaluronidase activity has been recognized by biochemical means previously (41). Molecular cloning of the hyaluronidase present in bee venom (17) revealed strong similarity to a previously known protein that is localized on the posterior head of human sperm, first characterized by means of a monoclonal antibody, and was therefore called PH-20 (14). PH-20 from man exhibits strong and robust hyaluronidase activity at neutral pH (18, 42, 43). As recently described, point mutations not only in *Spam1*, but also in other hyaluronidase-like genes such as *Hyal5* and *HyalP1* correlate with sperm dysfunction (26). As pointed out above, no further gene that potentially would encode hyaluronidase-like proteins others than HYAL1-4 and PH-20/SPAM1 has been discovered in the human genome to date. Besides mouse, *Hyal5* is also present in rat (accession number BC091219). This suggests that further gene duplication has occurred in rodents (26). Yet, mature sperm of SPAM1-deficient mice still exhibits hyaluronidase activity. It could be used to fertilize eggs (23) and is able to disperse cumulus cells from the cumulus mass (24). In light of our results, we assume that this is due to functionally active HYAL5 present on the surface of *Spam1* ^{-/-} sperm. We furthermore conclude, after having shown that SPAM1 as well as HYAL5 are functionally active hyaluronidases, that both enzymes participate in sperm fertilization of the egg.

The biological role for HYALP1 remains to be defined. SPAM1, HYAL5 and HYALP1 (39-64% identical; 64-81% similar) share also identical residues and a high degree of similarity with other well characterized hyaluronidases such as human PH-20 or bee hyaluronidase (supplementary Fig. 1). The most remarkable structural feature of hyaluronidase is a large groove (44) (Fig. 6). The amino acid residues of the groove that were found to come in close proximity to the substrate, are arranged in well-conserved sequence blocks, and, we thus consider them functionally relevant for proper positioning of the sugar polymer. In particular, we propose that four more amino acid moieties (indicated by x in Fig. 6A) that are located in close proximity to the active center, control the positioning of the substrate at the active

center. Two amino acid residues were found conserved in all three proteins (yellow-shaded x in Fig. 6A). The other two are different in HYALP1 (labelled green and blue in Fig. 6A). Molecular modelling revealed a close relationship between the geometries of the active centers of bee hyaluronidase, SPAM1 and HYAL5. However, major spatial differences were evident in the structure of HYALP1, in particular amino acid moieties that appear to be important for the proper positioning of the substrate with regard to the acidic amino acid moieties within the active center Fig. 6B). The active sites of these enzymes consist of aspartate and glutamate interspaced by one amino acid residue (45, 46), Notably, HYAL5 and SPAM1, yet also HYALP1 show this particular feature. Since both active center residues are present in HYALP1, we assume that the protein, though hydrolysis of HA was undetectable, might exhibit some yet unknown enzymatic activities. For the substrate binding groove of HYALP1 showed a high resemblance to the structures of bee hyaluronidase and the structural models of other known hyaluronidases, binding of HA or other GAG could indeed be possible. Human PH-20 exhibits many more functions besides HA degradation (19). Whether HYALP1 is functionally involved in metabolism of GAG or plays some role during sperm maturation will be investigated in future experiments.

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FIGURE LEGENDS

Fig. 1. Expression analysis. Hyal5 and Hyalp1 mRNA expression was determined in various tissues using Northern blot analyses (A), reverse transcription PCR (B) as well as quantitative PCR analysis (C). As a loading control, an image of the ethidium bromide stained agarose gel before transfer to the nylon membrane for hybridization with α ^{33}P labelled probe is shown in the lower part of panel A. For relative quantitation after PCR (B, C), template cDNAs were diluted with regard to β -actin mRNA content. Amplification cycles were either 30 or 40 times as indicated. After evaluation of quantitative PCR results, the transcript levels were further normalized to the respective mRNA content of Hyal5 or Hyalp1 in testis (C).

Fig. 2. Transcript localization in testicular tissue. (A) Histological section of murine testis stained with haematoxylin/eosin. Indicated is connective tissue between testicular tubules (ct), the lumen of a tubule (l), the cellular layer containing maturing spermatogenic cells (S) and the *lamina propria* (lp), the outer layer of tubules. (B-D) mRNA distribution of Hyal5, Hyalp1 and Spam1 in testicular tissue was determined by in situ hybridization on histological sections either with antisense or sense probes (inlaid micrographs). Digoxigenin-labelled antisense RNA probes were detected with the aid of anti-digoxigenin Fab alkaline phosphatase complex and BM Purple (Roche) as substrate. Bars indicate 100 μm .

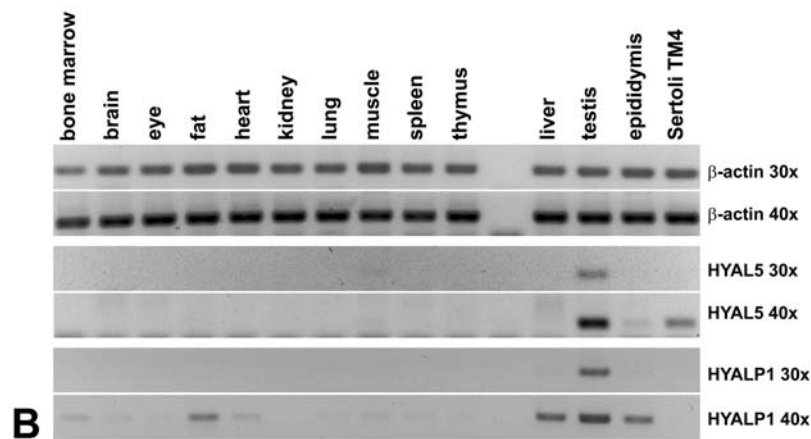
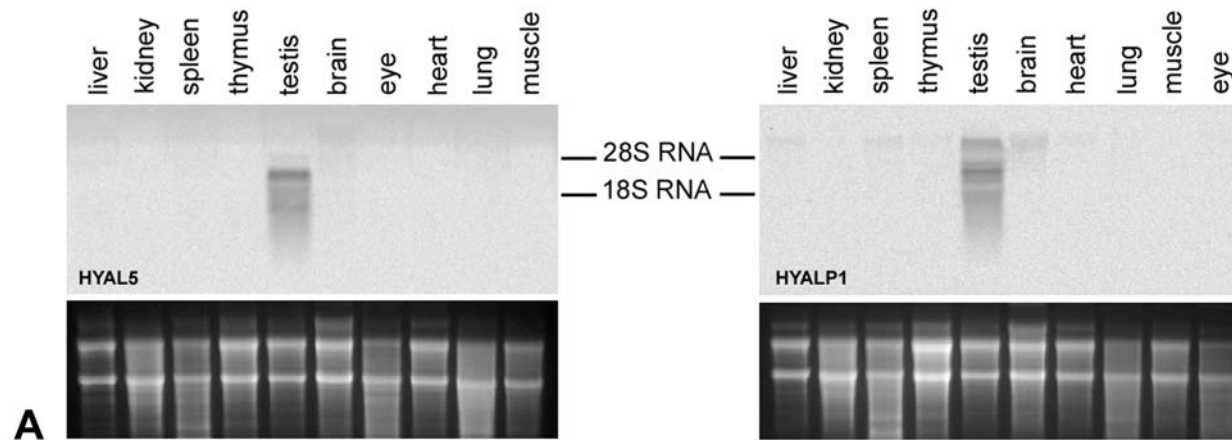
Fig. 3. Hyaluronidase activity of HYAL5 and HYALP1 expressing *Xenopus laevis* oocytes. Oocytes injected with cRNA encoding SPAM1, HYAL5 or HYALP1 and uninjected controls (oocyte) were cultured in 200 μl O-R2 buffer for 24 h. Homogenized oocytes expressing hyaluronidase-like proteins were incubated with 20 ng of 5-aminofluorescein-labelled HA for either 16 hours or 49 hours, respectively. Hyaluronidase activity was tested at different pH values as indicated.

Fig. 4. *In vitro* translation of Hyal5 and Hyalp1 cRNA. After protein synthesis with the aid of a cell-free reticulocyte system (Novagen), sulphur-35 labelled protein products were separated by SDS-PAGE and detected by means of fluorography and phospho-imaging.

Fig. 5. Subcellular localization of HYAL5 and HYALP1. Hyal5, HyalP1 and Spam1 cRNA, respectively, were translated in intact *Xenopus* oocytes. Oocytes were incubated for two days in O-R2. Subsequently, healthy oocytes were subjected to phospholipase C (PLC) treatment (indicated by +) or phospholipase buffer without enzyme (-). The supernatant of these reactions was mixed with fluorescein-labelled HA and soluble hyaluronidase activity was determined using standard conditions at pH 6.5.

Fig. 6. Structural models: (A) Mouse hyaluronidase-like protein sequences, SPAM1 (accession number AAP49832), HYAL5 (accession number BAC55071) and HYALP1 (accession number BAB30316) were pre-aligned using ClustalW and the resulting alignment was finally adjusted manually. Amino acid residues identical in all three sequences were box-shaded.

(B) On the basis of the X-ray structure of bee hyaluronidase (PDB accession code: 1fcv), 3D-structural models for SPAM1, HYAL5 and HYALP1 were generated (upper row). Residues that were marked with ○, ●, ■ and ▲ and marked as such x in A, were found to be located opposite and in close proximity to the active center (ac) within the tapering substrate binding cleft. These appear to directly interact with the substrate. The 3D-models were slabbed along the line drawn in the frontal view of the bee hyaluronidase structure. Surfaces were drawn in light grey, interior parts of the molecules in dark color. Side projections of the active center reveal variant geometries of the active centers of active hyaluronidases, (bee hyaluronidase, HYAL5 and SPAM1) and inactive HYALP1.



C

	relative abundance	
	HYAL5	HYALP1
testis	100	100
epididymis	1.23	1.15
Sertoli	0.68	0.02
other tissues	< 0.56	< 0.70

