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Mouse Spam1 (PH-20) Is a Multifunctional Protein: Evidence for Its Expression in the Female Reproductive Tract[1]

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ABSTRACT

Sperm adhesion molecule 1 (Spam1) is a widely conserved sperm surface protein with multiple roles in mammalian fertilization. Although the gene for this protein has been thought to be testis specific based on Northern blot analysis, there is evidence for nontesticular expression when transcripts are analyzed by more sensitive techniques. In the present investigation, results of a reverse transcription polymerase chain reaction assay, an RNase-protection assay (RPA), and an in situ transcript hybridization assay revealed that the murine *Spam1* gene is transcribed in the female genital tract. RPA revealed that *Spam1* transcripts are synthesized in a region-dependent manner, with the oviduct having lower transcript levels than the uterus and vagina. The transcripts levels were 3- to 10-fold lower in the female genital tract than in the testis. In situ transcript hybridization assay revealed RNA in the luminal epithelium in all three regions of the genital tract and in the uterine myometrium and the oviductal mesothelium. Western blot analysis and immunohistochemistry demonstrated that the protein concentration is 1.5- to 3-fold lower in female tissues than in sperm, and localization is similar to that of the transcripts. The protein has hyaluronidase activity at neutral pH, which is unique for sperm hyaluronidase, but not at acidic pH. In the uterus, Spam1 expression fluctuated during the estrous cycle. Its localization suggests that in addition to functioning as a secretory protein, it may be involved in hyaluronic acid metabolism or turnover in the female genital tract. Our results provide further evidence that Spam1 is a multifunctional protein and that it is less restricted in its expression than previously reported.

female reproductive tract, fertilization, testis, uterus, vagina

INTRODUCTION

Sperm adhesion molecule 1 (Spam1 or PH-20) is the widely conserved sperm-surface hyaluronidase [1–3]. Unlike ubiquitous hyaluronidases, which are active only at acidic pH [4], sperm hyaluronidase is unique among hyaluronidases because it is active at neutral and acidic pHs [5]. These different enzymatic activities are attributed to the insoluble and soluble forms, respectively, of the protein and arise from two different catalytic sites within the hyaluronidase domain, which is located at the N-terminal end of the molecule [5]. The hyaluronidase domain also contains a hyaluronic acid (HA) binding site that plays a role in the signaling event leading to acrosomal exocytosis [6]. The other functional domain of the protein is the zona-binding domain, which is found at the C-terminal end of this single-subunit glycosyl phosphatidylinositol (GPI)-linked molecule. Thus, the protein is currently known to play at least three roles in mammalian fertilization.

Northern blot analysis has revealed that Spam1 protein is testis specific in humans [7], guinea pigs [8], and rats and mice, where spermatids are the expressing cell type [9, 10]. In the mouse, Spam1 protein is also expressed in the epithelium of all three regions of the epididymis, as shown in sperm-free mutant and wild-type mice [11]. In these animals, *Spam1* transcripts were detected in the epididymis using the more sensitive techniques of in situ transcript hybridization [11] and RNase protection assay (RPA) (unpublished data). Spam1 expressed in the epididymis has hyaluronidase activity only at neutral pH, which is typical of the insoluble form [11], and the protein is secreted [12] in the form of membrane-bound vesicles in the luminal fluid [13] and in the medium of cultured epithelial cells. Epididymal and testicular Spam1 are differentially regulated, as indicated by different mRNA transcription start sites [12] and different average lengths of poly(A) residues [13]. Additionally, the proteins from these two organs have different glycosylation patterns, producing different isoforms. This finding indicates that there are tissue-specific posttranslational modifications of Spam1 [13].

The expression of Spam1 in the epididymis led us to the hypothesis that the protein plays a role in sperm maturation. This hypothesis is supported by the observation that epididymal Spam1 is secreted with its lipid anchor, a form in which it can bind to sperm [13], possibly via exosomes or epididymosomes as shown for other GPI-linked epididymal proteins [14]. The protein is also expressed in the vas deferens of the mouse (unpublished results), a finding that further supports the role of Spam1 in sperm maturation, which has been attributed to this organ [15]. The finding that Spam1 is found along the extratesticular pathway of the mouse in a form in which it can bind to sperm led us to hypothesize that it is also secreted along the female reproductive tract, which is traversed by sperm. This situation would be similar to that of the porcine spermadhesin genes, which are expressed in both the male and female reproductive tracts [16]. The purpose of this investigation was to determine whether *Spam1* is expressed in the female genital tract and, if so, the characteristics of its expression in this system. We found that Spam1 is synthesized in the female genital tract, where the protein has hyaluronidase activity at neutral pH and where it may function as a secretory protein and may be associated with HA turnover in the myometrium.

MATERIALS AND METHODS

Animals and Reagents

Sexually mature female ICR outbred or C57BL/6N mice (Harlan Sprague-Dawley, Indianapolis, IN) were used in all studies. The studies

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were approved by the Animal Care 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). All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific Company (Malvern, PA) unless otherwise specified.

Preparation of Total RNAs from Vagina, Uterus, and Oviduct

Total RNAs from vagina, uterus, and oviduct of six to eight females were extracted using Tri-Reagent according to the manufacturer's protocol. RNA samples were further treated with RNase-free DNase (Boehringer Mannheim, Indianapolis, IN) (final concentration of 5–10 U/ml) for 2 h at 37°C, followed by phenol/chloroform extraction and ethanol precipitation.

Reverse Transcription Polymerase Chain Reaction

First strand *Spam1* cDNA synthesis from 2 μg of total RNA was performed with a SuperScript Preamplification System (Gibco, Rockville, MD) under the conditions recommended by the manufacturer. Control experiments were performed simultaneously without the addition of reverse transcriptase. One microliter of each reverse transcription (RT) product was subjected to polymerase chain reaction (PCR) amplification using a pair of primers designed from the 3′ untranslated region (UTR) of the mouse *Spam1* cDNA sequence (GenBank accession U33958): forward primer, nucleotides (nt) 1705–1725; reverse primer, nt 2026–2046. The PCRs were performed under the following conditions: 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 60°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. Total RNAs extracted from testis and muscle were used along with the samples as positive and negative controls, respectively. The PCR products were resolved in a 1% agarose gel and stained with ethidium bromide. All experiments were repeated.

Sequencing of the RT-PCR Products

The RT-PCR products were cloned into pCR 4-TOPO TA vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Several clones were isolated and sequenced in our core facility.

In Situ Transcript Hybridization

Antisense and sense RNA probes generated from PCR of a 342-base pair (bp) fragment (nt 1705–2046) from the 3′ UTR of the murine *Spam1* were labeled with digoxigenin-11-UTP (DIG) using an in vitro transcription Riboprobe System (Promega, Madison, WI) in accordance with the manufacturer's protocol. Paraffin-embedded tissue sections were dewaxed and fixed for 30 min in 4% paraformaldehyde in diethyl pyrocarbonate (DEPC)-treated PBS, washed twice for 15 min in PBS containing 0.1% active DEPC, and equilibrated for 15 min in DEPC-treated 5× saline sodium citrate (SSC). Sections were prehybridized for 2 h at 54°C in 500 μl hybridization buffer (50% formamide, 5× SSC, 40 μg/ml salmon sperm DNA). The probes were denatured by heating to 100°C for 5 min and rapidly plunged into an ice bath for 30 sec. They were then diluted to 400 ng/ml in hybridization buffer and applied to the slides with the sections for 40 h at 54°C for hybridization.

After hybridization, slides were washed for 30 min in 2× SSC at room temperature, 10 min in 2× SSC at 65°C, 10 min in 1× SSC at 65°C, and 10 min in 0.1× SSC at 65°C. Slides were equilibrated for 5 min in TBS, pH 7.5 (100 mM Tris, 150 mM NaCl), and then incubated for 2 h with alkaline phosphatase (AP)-anti-DIG antibody (Roche, Indianapolis, IN) diluted 1:300 in TBS containing 1% BSA (w/v). Slides were then washed twice for 15 min in TBS and equilibrated for 5 min in TBS, pH 9.5 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$). The signal was visualized by incubation with nitroblue tertrazolium/5-bromocresyl-3-indolylphosphate solution (Pierce, Rockford, IL) for 15–30 min (until a dark purple color was visible). The reaction was stopped by rinsing with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 15 min. Slides were then rinsed in water, counterstained in 1% neutral red, dehydrated in an ethanol series, cleared in xylene, and mounted in permount. Imaging was done using an Axioskop (Zeiss, Oberkochen, Germany) and an MDS digital camera (Kodak, Rochester, NY).

RNase Protection Assay

Synthesis of an antisense RNA probe was performed as described by Zheng and Martin-DeLeon [10]. *Spam1* cDNA cloned in the pMAL-p vector was cut with the *Stu*1 restriction enzyme to obtain a linearized template. Antisense RNA probe was transcribed using T3 polymerase to generate a ^{32}P-labeled fragment complementary to the terminal 230 nt in the *Spam1* 3′ UTR. Hybridization and RNase digestion reactions were done using the RPA III Kit (Ambion, Austin, TX). Ten micrograms of RNA and 5×10^4 cpm of probe were coprecipitated with ethanol and resuspended in 10 μl hybridization buffer. The mixture was heated to 90°C for 4 min and incubated at 42°C for 24 h. After hybridization, 150 μl of RNase solution (RNase A/RNase T1 mix, diluted 1:100 in the RNase digestion buffer) was added, and the mixture was incubated at 37°C for 30 min. Digestion was terminated, and RNA was precipitated by RNase inactivation/precipitation solution. Protected probes were resolved on a 5% acrylamide/8 M urea denaturing gel, which was exposed to autoradiographic film for 12 h. An RPA using an antisense β-actin probe was performed as an internal control. This probe protects a 245-nt region of β-actin cDNA.

Northern Blot Analysis

Northern blot analysis was performed using total RNA and poly(A) mRNA as previously reported [10].

Preparation of Protein Extracts from Vagina, Uterus, and Oviduct

Protein extracts, obtained for three experiments with three or four animals each, were prepared by manually homogenizing the tissues (using a mortar and pestle) with a solubilization buffer (62.5 mM Tris-HCl, 10% glycerol, 1% SDS, 1 mM PMSF, pH 6.8) at 4°C. The suspension was centrifuged at $10\,000 \times g$ for 10 min at 4°C, and the supernatant containing the proteins was collected. The protein concentrations of all samples were determined with a biocinchoninic acid protein assay kit (Pierce), using different concentrations of BSA as standards. An equal mass (60 μg) of each protein sample was used for all experiments.

SDS-PAGE and Western Blot Analysis

Each protein sample was exposed to reducing conditions (heated at 99°C for 4 min in the presence of 100 mM dithiothreitol), subjected to 15% SDS-PAGE, and transferred to a nitrocellulose membrane according to standard protocols. Protein extracts from caudal sperm and muscle were analyzed as positive and negative controls, respectively, along with the samples. Western blotting was performed with the WesternBreeze Chemiluminescent Immunodetection Kit (Invitrogen) according to the manufacturer's protocol. The membrane was blocked for 30 min at room temperature and then probed with rabbit antipeptide Spam1 antiserum [11] (Zymed, South San Francisco, CA), diluted 1:1000 in blocking solution. The antipeptide antiserum was generated from a 15-mer oligopeptide (C-terminal 381–395) specific for Spam1 [11]. The membranes were washed, and the secondary antibody, AP-conjugated anti-rabbit IgG, was applied. The protein was visualized using the chemiluminescence substrate provided with the kit. To determine equal loading α-tubulin was used as an internal control.

HA Substrate Gel Electrophoresis

Hyaluronidase activities in tissues were measured using HA substrate gel electrophoresis (HASGE) performed as described by Guntenhoner et al. [17] and Deng et al. [11]. HA from bovine vitreous humor was added to a 15% SDS-polyacrylamide gel at a final concentration of 0.15 mg/ml. Gels were run at 15 mA constant current until the leading dye (bromophenol blue) had migrated near the bottom. After electrophoresis, gels were incubated at room temperature for 2 h in PBS containing 3% Triton X-100 on a rocking platform to remove SDS. Gels were then incubated in 100 mM sodium acetate (pH 7.0) at 37°C for 24–48 h. To visualize the digestion of the HA, gels were stained with 0.5% Alcian blue in 3% acetic acid for at least 2 h, destained in 7% acetic acid, and then counterstained with Coomassie brilliant blue G-250. Undigested HA is stained with Alcian blue and shows a dark blue background against an unstained area with digested HA. Images showing the digestion of the HA by hyaluronidase activity in each sample were captured by scanning the gels with a laser densitometer. Protein extracts from caudal sperm and muscle samples were used as positive and negative controls, respectively, and were ana-

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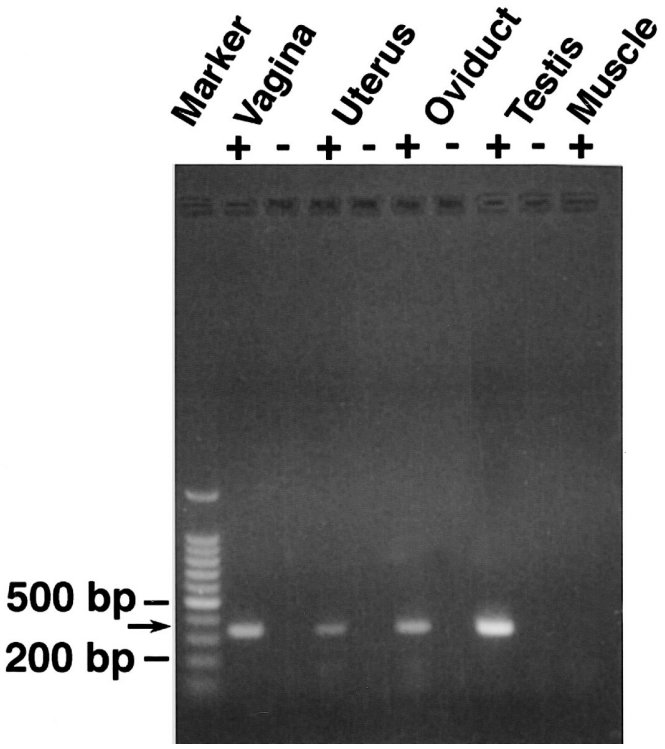


FIG. 1. The presence of steady-state *Spam1* mRNA as detected by RT-PCR of the total RNAs from mouse vagina, uterus, and oviduct. Two micrograms of RNA was subjected to first-strand synthesis with (+) or without (−) reverse transcriptase followed by PCR amplification. Products of the expected size were obtained for all RNA except muscle (the negative control). The marker, a 100-bp ladder, is shown on the left. The same results were obtained for replicate experiments.

lyzed along with the samples from the reproductive tract to determine their ability to digest HA.

Immunohistochemistry and Confocal Fluorescence Microscopy

Vagina, uterus, and oviduct were fixed in 10% (v/v) neutral buffered formalin. Paraffin-embedded cross sections were made according to standard protocol. Prior to immunodetection, the slides were dewaxed four times for 5 min each with xylene and then rehydrated by incubation in 100%, 90%, 80%, 70%, and 50% ethanol for 5 min each. The slides were stained with 0.5% toluidine blue for 5 min to block autofluorescence from tissues, refixing with 4% (w/v) paraformadehyde in 1× PBS (pH 7.5) for 1.5 h at room temperature, and then blocked for 1 h with 2% (w/v) BSA in 1× PBS (pH 7.5).

One hundred microliters of the rabbit antipeptide mouse Spam1 antiserum (diluted 1:400 in 1× PBS containing 2% BSA) was added to each slide. The slides were then incubated in a humid atmosphere for 1 h at room temperature. After three 5-min washes with 1× PBS, slides were incubated with the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (diluted 1:320 in 1× PBS containing 2% BSA), for 30 min at 4°C. Control slides were incubated with preimmune rabbit serum as the primary antibody followed by detection with the FITC-conjugated secondary antibody. After three washes for 5 min each in 1× PBS, the slides were mounted in ρ-phenylenediamine (1 mg/ml) antifade. The specimens were examined and imaged with a Zeiss LSM 510 NLO multiphoton confocal microscope using the 488-nm and 647-nm lines of the Ar/Kr laser for FITC.

Analysis of Spam1 Expression During the Estrous Cycle

A total of 11 sexually mature (8–12 wk old) virgin female mice were used in the study. Females were categorized by estrous cycle stage based on vaginal smears and were grouped for the collection of tissues. Tissues were obtained from three females each at diestrus, proestrus, and estrus and from two females at metestrus. Tissues were then processed for the analysis of mRNA and protein expression by in situ hybridization and immunohistochemistry, respectively.

Statistical Analysis

Results are expressed as mean and SEM. Data from testis and female reproductive tract were analyzed using a *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

The use of specific primers for *Spam1* RT-PCR analysis of the mRNA from the murine female reproductive tract resulted in a 342-bp product from the vagina, uterus, and the oviduct in tissues of virgin females (Fig. 1). This product is identical in size to the expected product from the testis (Fig. 1). Cloning and sequencing of the product showed complete sequence identity with *Spam1* mRNA originating from the testes. No PCR product could be generated in the absence of RT in the samples from the female tract, indicating the absence of DNA contamination in the samples. No product was obtained from mRNA extracted from skeletal muscle, which was used as a negative control. The presence of *Spam1* transcript in the female reproductive tract was confirmed by the less sensitive RPA (Fig. 2A). Expression along the female tract was region dependent, with the level increasing from the oviduct to the va-

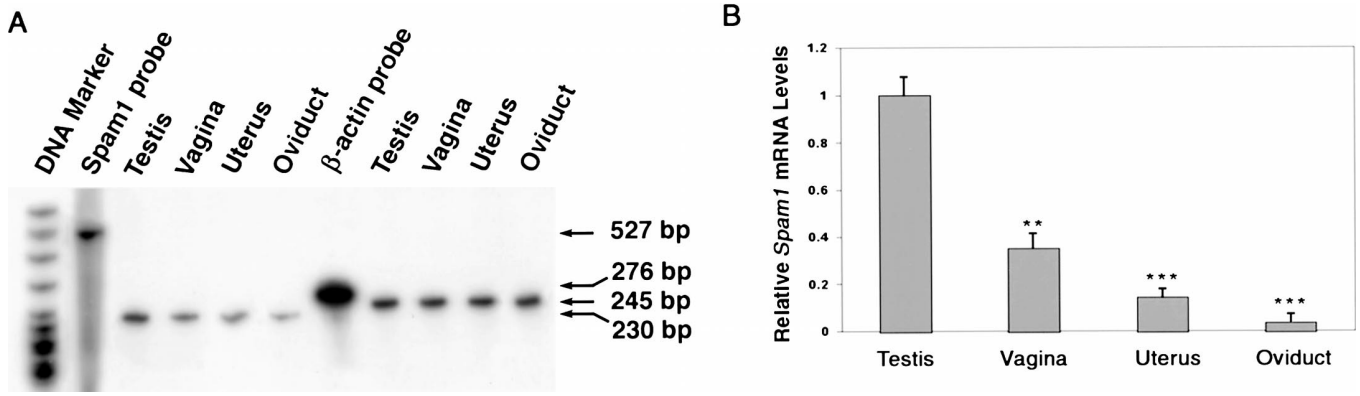


FIG. 2. Autoradiography for RPA of RNA from testicular and female reproductive tissues. The antisense *Spam1* probe is complementary to the terminal 230 nt in the 3′ UTR. β-Actin was used as an internal control, and this probe protects a 245-nt fragment of the transcript. **A**) Gel representative of three independent experiments. **B**) Relative levels of mRNA, as assessed by laser densitometry. Values are mean ± SD, with significant differences from testis indicated (**$P < 0.01$ or ***$P < 0.001$).

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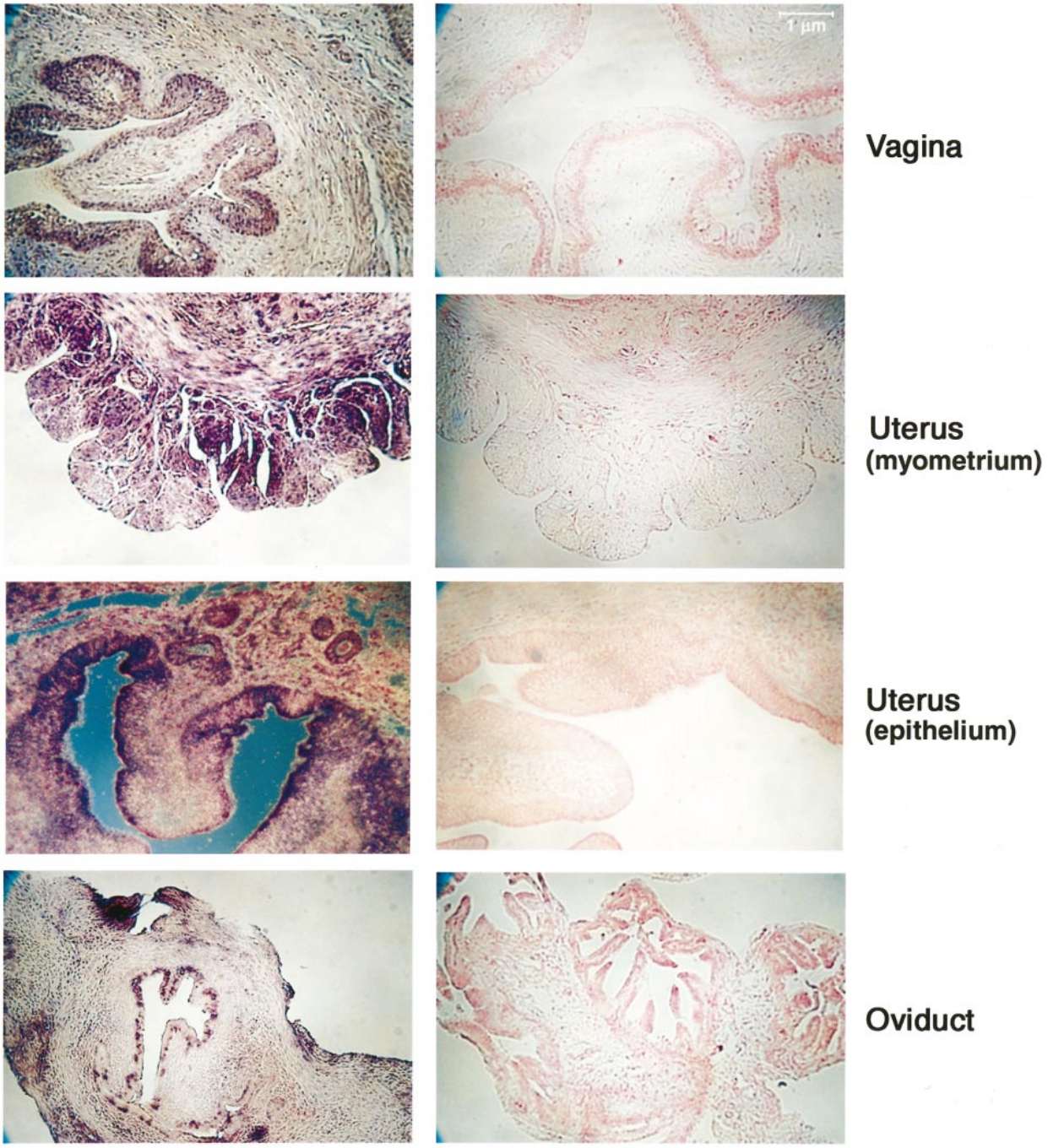


FIG. 3. In situ hybridization localization of *Spam1* transcripts in mouse vagina, uterus, and oviduct. Histological sections hybridized with an antisense *Spam1* RNA probe, showing on the left the positive purple staining in the luminal epithelium in all three regions. Along with staining in the luminal epithelium of the uterus, there is staining of the glandular epithelium as seen in the section taken from an animal in proestrus. The uterine section showing staining in the myometrium was obtained from an animal in estrus. On the right are control sections from all three regions hybridized with the sense probe showing only the neutral red staining. Using tissues from different mice, the same results were obtained for replicate experiments. Bar = 1 μm for each plate.

gina. After comparing the equal intensity of the 245-nt protected β-actin fragment (internal control) in all organs, the abundance of *Spam1* transcripts in the female genital tract was estimated to be approximately 3- to 10-fold lower than that in the testis (positive control) (for mean ± SD, see Fig. 2B).

To determine the location of *Spam1* mRNA synthesis, in situ transcript hybridization was performed. Transcripts were detected in the epithelium of the vagina and the oviduct and in the uterus in both the luminal and glandular epithelia, the myometrium, and possibly the stroma, as indicated by the purple stain (Fig. 3). Control sections that were treated with the sense riboprobe showed only the neutral red staining. We also investigated whether the level of the transcript in the female genital tract could be determined by Northern blot analysis. Using both total and poly(A) mRNA, no band was detected for any of the regions in the female tract, but the characteristic 2.1-kilobase

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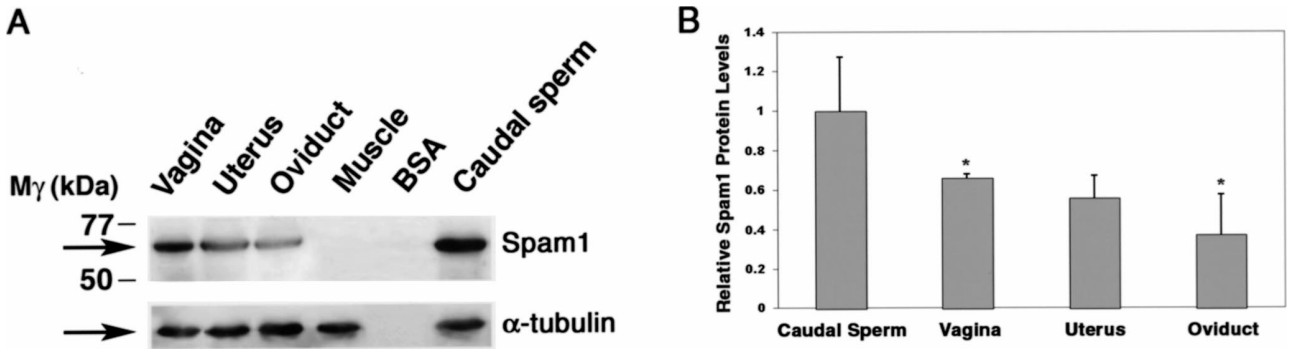


FIG. 4. Western blot analysis showing the presence of Spam1 protein in the female genital tract and in caudal sperm. Protein extracts from skeletal muscle and BSA served as negative controls. In each lane, 60 μg of total protein was loaded. Equal loading is seen using the α-tubulin control. **A**) Gel representative of three independent experiments. **B**) Relative amounts of protein in sperm and in the three regions in the female tract. Values are mean ± SD, with significant difference from testis indicated (*$P < 0.05$).

testicular band [10] was present when equal amounts of mRNA were loaded, as detected by probing with β-actin (data not shown).

Western blot analysis of tissue extracts revealed that the 67-kDa Spam1 protein is present in the female tract (Fig. 4A). A comparison of the protein in sperm cells with that from the female tissues showed relative levels (Fig. 4B) that were 1.5- to 3-fold lower in the female (for mean ± SD, see Fig. 4B). The pattern of expression for the different regions paralleled that for the transcripts detected with the RPA (Fig. 2). No protein was seen for the two negative controls, skeletal muscle and BSA. Spam1 in the female tract had hyaluronidase activity only at neutral pH, as revealed by HASGE with a 67-kDa band that was unstained (Fig. 5B). However, testis showed activity at both neutral and acidic pH (Fig. 5, A and C). The relative levels of hyaluronidase activity at pH 7.0 in the three regions of the female tract were approximately 1.4- to 3.0-fold that in sperm, corresponding to the protein levels (for mean ± SD, see Fig. 5B). No enzymatic activity was observed in skeletal muscle and BSA, as expected.

Immunohistochemistry showed that Spam1 protein is localized in the epithelium and lumen of the vagina (Fig. 6). In the uterus, strong immunoreactivity was found in the epithelium and the myometrium, and in the oviduct staining was seen along the luminal epithelium and on the outer lining. In general, these locations are parallel to those detected for the mRNA using in situ transcript hybridization.

To determine the uterine expression pattern in cycling females, vaginal smears were examined to determine estrous cycle stage. Uterine tissues collected from virgin cycling females at the four stages of the estrous cycle showed a similar pattern of staining when subjected to analysis by in situ transcript hybridization and immunohistochemistry. Figure 7 shows the immunolocalization of the protein at the various stages of the cycle. At diestrus, the luminal epithelium and the myometrium showed minimal staining (Fig. 7). At proestrus and estrus, both the luminal and the glandular epithelia and the myometrium stained positively for both the RNA and protein (Figs. 3, 6, and 7). However, no staining was detected at metestrus in either the luminal epithelium or the myometrium (Fig. 7).

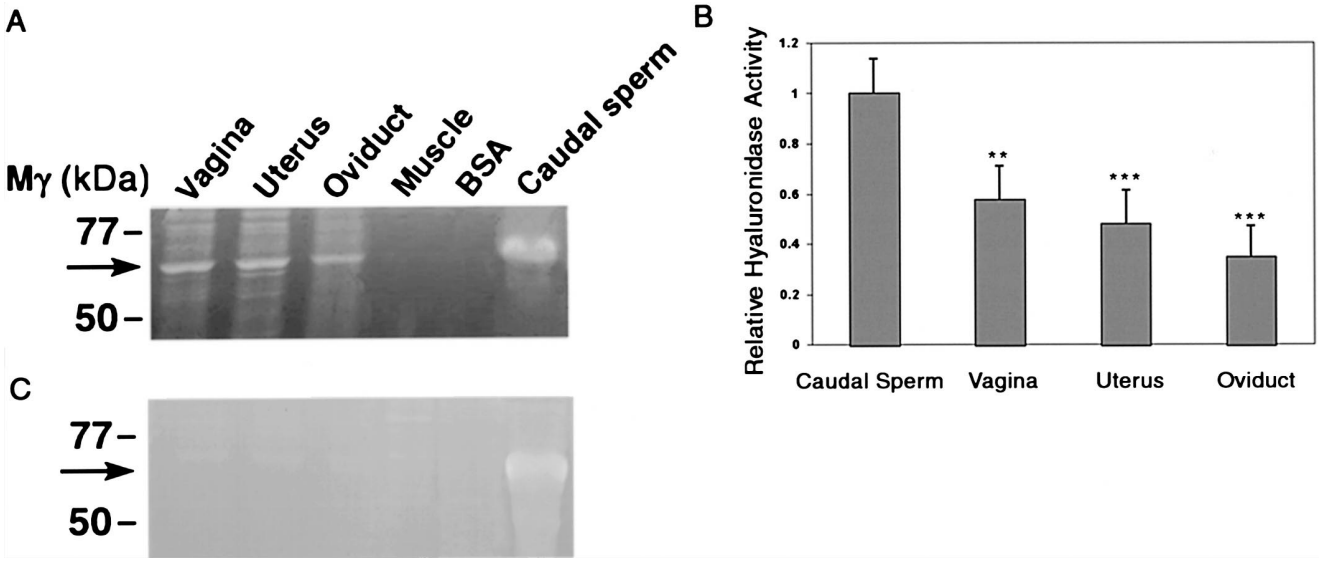


FIG. 5. HASGE of protein extracts from the female genital tract, caudal sperm, skeletal muscle, and BSA. In each lane, 60 μg of total protein was loaded, and the assays were performed at neutral pH (7.0) (**A**) and at acidic pH (4.0) (**C**). The gels are representative of three independent experiments. Relative hyaluronidase activity of Spam1 in the female genital tract and caudal sperm (**B**) was determined by analyzing HA substrate gels with laser densitometry. The result is the mean of the data obtained from 18 sexually mature ICR outbred or C57BL/6N mice (nine females and nine males). Values are mean ± SD, with significant differences from testis indicated (**$P < 0.01$ or ***$P < 0.001$).

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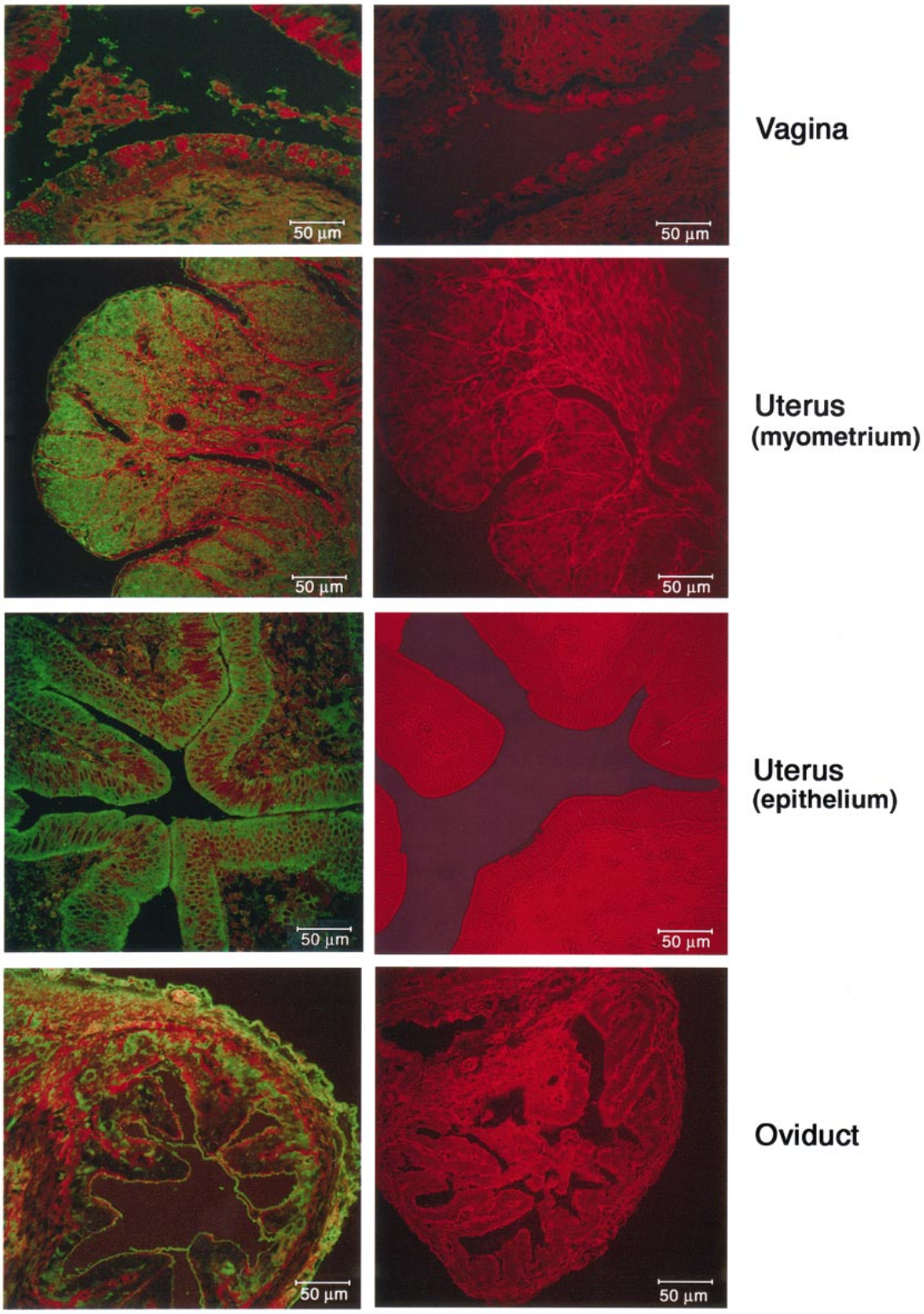


FIG. 6. Immunohistochemical localization of mouse Spam1 using multiphoton confocal microscopy. On the right are control sections treated with preimmune serum and the FITC-conjugated secondary antibody. These sections are from the red confocal channel, showing the morphology of the tissue while eliminating the effect of autofluorescence. These sections show no green staining, unlike those on the left that are treated with the antipeptide of Spam1 as the primary antibody. In the vaginal and oviductal lumen, positive green staining material is visible along with that in the epithelia. Positive staining is also visible in the myometrium and the outer covering of the oviductal wall or the mesothelium. Bar = 50 μm.

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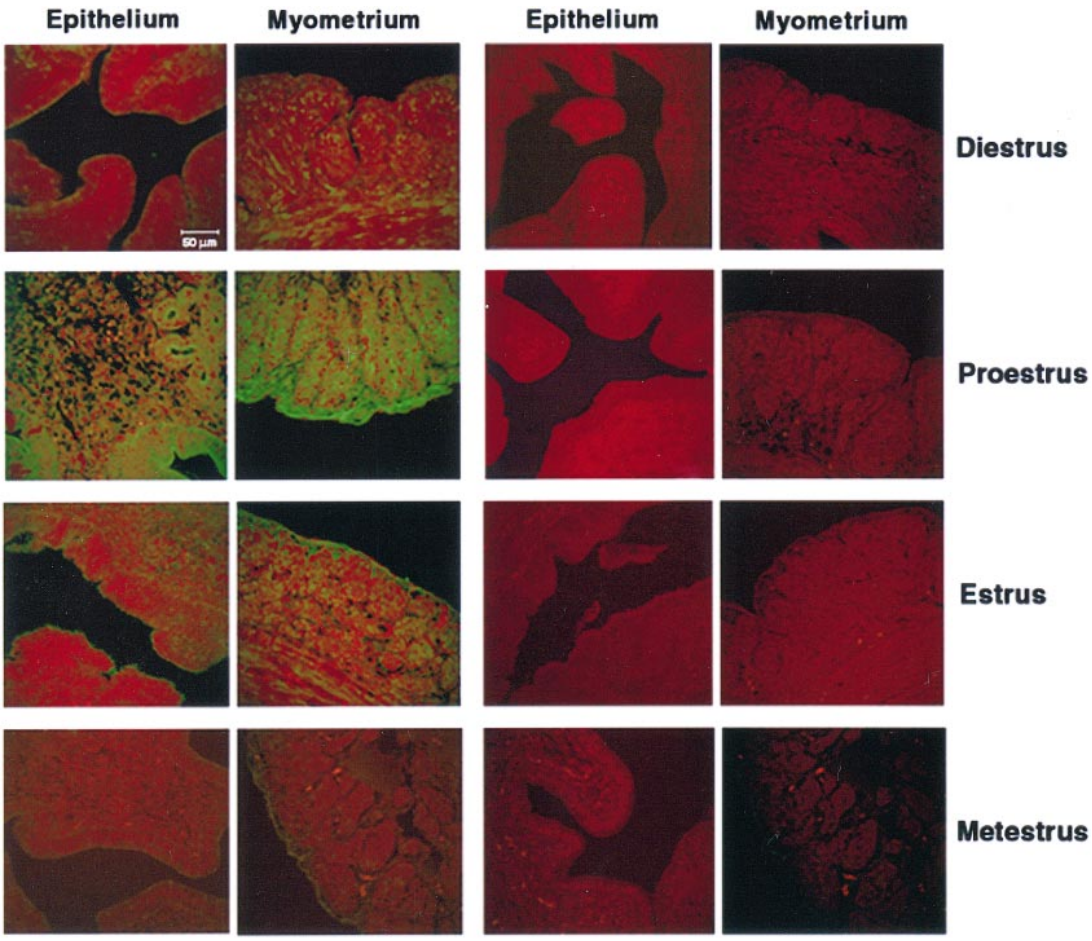


FIG. 7. Immunhistochemical localization of Spam1 in mouse uterus during the estrous cycle. The staining pattern is shown for both the luminal epithelium and the myometrium. The treatment of the tissues and the imaging are as described in Figure 6, and test samples and controls are on the left and right, respectively. Bar = 50 μm (for each plate). In proestrus, in addition to the luminal epithelium, the glandular epithelium is also positively stained, as is the case for the mRNA in Figure 3.

DISCUSSION

In this study, different regions of *Spam1* cDNA were used to generate riboprobes specific for *Spam1* that would not hybridize with sequences homologous to other hyaluronidases. The antibody used in the detection of the protein is a polyclonal antipeptide derived from a 15-mer at the C-terminal end (residues 381–395) that is unique for Spam1 [11]. Using three methods for mRNA detection and two immunological approaches for the detection of the protein, we clearly showed that *Spam1* is transcribed and the protein is synthesized in the murine female reproductive tract.

Although the level of mRNA in the testis, which was used as a positive control, was robust and could be detected by Northern blot analysis, this was not the case for tissues from the female reproductive tract. This finding is similar to that reported for humans, where the screening of multiple tissue cDNA libraries showed that *SPAM1* expression is detectable by Northern blot in the testis but only by RT-PCR in prostate, fetus. and placenta [18]. There are two possible explanations for the different levels of expression in testicular and nontesticular tissues. A more robust transcription of *Spam1* mRNA may occur in the testis because of the presence of testis-specific transcriptional factors such as cAMP-responsive element modulator (and its activator, ACT) [19] whose binding site, cAMP-responsive element, is present in the proximal promoter [20]. Based on findings in the epididymis, transcripts in the testis may be more stable than nontesticular transcripts because of the increased length of their poly(A) tails [13], which is likely to be mediated by the recently discovered testis-specific polyadenylation polymerase [21].

In our study, steady-state levels of *Spam1* mRNA detected by RPA showed that female reproductive tissues, with their rare transcripts, have 3- to 10-fold lower transcript levels than the testis, where transcripts are abundant. However, female transcripts, unlike testicular transcripts, were not detected by Northern blotting. This discrepancy in the detection of the mRNA is due to the fact that Northern blotting is less efficient for the detection of rare messages because cDNA-RNA hybrids dissociate during the washing steps. However, RPA is effective in detecting rare messages via RNA-RNA hybrids, which are analyzed in the absence of a washing step.

Greater degradation of the transcript in females would not necessarily affect the levels of the protein, which is translated immediately after transcription [22] and is highly stable, appearing on mature sperm in the caudal epididymis after synthesis weeks earlier in spermatids in the testis [22]. The levels of protein in the female tract were only 1.5- to

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3-fold lower than that in sperm in a comparison between cells in the male and tissues in the female. Because in the female the Spam1-expressing cell type accounts for only a fraction of the cell population, the relative level of the protein in the female compared with sperm would be expected to be lower, not higher, than the 3- to 10-fold lower values seen for the mRNA comparison in the testis. The higher concentrations of the protein detected are consistent with the rapid degradation of the transcript in the female.

In the vagina, the protein was detected on the supranuclear region of the surface epithelium and as secretory material in the vaginal lumen of virgin animals. Similarly, in the uterus and oviduct Spam1 was also found in the luminal epithelium, the myometrium, and the outer covering of the oviduct. Although the protein expressed in all three regions of the genital tract demonstrated hyaluronidase activity at neutral pH, which is unique for sperm-surface hyaluronidase, there was no activity at acidic pH. This situation is similar to that for epididymally expressed mouse Spam1 [11, 12]. However, sperm Spam1 demonstrates enzyme activity at both neutral and acidic pH, with the latter generally detected in the soluble form of the protein, which is released after cleavage from the membrane during the acrosome reaction [23].

The relative levels of hyaluronidase activity of Spam1 in the female tract ranged from 1.4- to 3.0-fold that in sperm. This finding is similar to that for the protein and corroborates the finding of region-dependent expression of protein along the genital tract, with the lowest activity seen in the oviduct and the highest in the vagina. The correlation of the levels of Spam1 protein and its hyaluronidase activity in the female tract and in sperm suggests that Spam1 from the two sources may have similar patterns of *N*-linked glycosylation, which in the mouse and horse is associated with SPAM1 hyaluronidase activity [24, 25] and shows tissue-specific differences [26]. Additional studies are required to determine the comparative distribution of *N*-linked glycosylation in the murine Spam1 from sperm and the female reproductive tract.

The results of in situ transcript hybridization assay and immunohistochemistry indicate hormone cycle dependency in the expression of Spam1 in the uterus. Although strong staining was observed in the uterine glands, the luminal epithelium, and the myometrium during estrus and proestrus, staining was completely absent during metestrus and began to rise during diestrus, suggesting a functional relationship. To further determine whether *Spam1* expression may be related to the estrous cycle, we searched a 2-kilobase region from the NCBI database upstream from the transcriptional start site to determine the presence of an estrogen receptor element. Using the DNASIS Mac version 3.5 program, a sequence at −457 to −438 was found with 60% homology to the consensus sequence (5′-AGGTCAnnnTGACCT-3′) that is the binding site for the estrogen receptor. The 5′ half-hexamer preceding the nnn section of the sequence has a homology of 75%, there are no mutations in either hexamer, and the specific central two (internal) nucleotides of each hexamer, GT and AC, are intact. It remains to be shown whether this sequence is a functional transcription factor binding site.

The finding of the protein in both the luminal epithelium, where it is likely to be secreted, and in the myometrium suggests that it may be playing at least two different roles in the female genital tract. As a secretory protein of the glandular and surface epithelium of the uterus and the oviductal and vaginal epithelia, Spam1 may impact sperm in two ways. As a hyaluronidase and a well-known glycosidase [27], Spam1 may serve to modify existing sperm surface proteins so that they become fully functional at the site of fertilization. Second, Spam1 from the female genital tract may directly bind to the sperm surface to replenish what may be lost in their journey through the tract. It is therefore important to determine whether Spam1 is present in the uterine luminal fluid and whether it is released with its lipid anchor, as occurs for the epididymal epithelium [13]. Glycoproteins that are secreted in vesicles with their lipid anchor have been shown to bind to sperm via exosomes [14].

In the myometrium and outer lining of the oviductal walls, Spam1, as a cell-surface adhesion molecule, may mediate binding to extracellular matrices via its hyaluronan binding motifs, as is the case for other glycoproteins such as CD44, RHAMM, and link protein [28]. HA is a potent bioactive macromolecule involved in many cellular functions, including the regulation of cytokines by human uterine fibroblasts [29]. The HA-binding receptor on Spam1 and/or its hyaluronidase activity may participate in the metabolism and turnover of HA that occurs physiologically in the female genital tract. Our findings therefore support and extend the notion that SPAM1 (PH-20) is a multifunctional protein. They also provide evidence that *SPAM1* is less restricted in its expression than previously thought and that the relative levels of its nontesticular to testicular protein expression are markedly higher than that of the mRNA.

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REFERENCES

1. Gmachl M, Kreil G. Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm. Proc Natl Acad Sci U S A 1993; 90:3569–3573.
2. Lin Y, Mahan K, Lathrop WF, Myles DG, Primakoff P. A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. J Cell Biol 1994; 125:1157–1163.
3. Hunnicutt GR, Primakoff P, Myles DG. Sperm surface protein PH-20 is bifunctional: one activity is a hyaluronidase and a second, distinct activity is required in secondary sperm-zona binding. Biol Reprod 1996; 55:80–86.
4. Csoka TB, Gregory IF, Wong T, Stern R. Purification and microsequencing of hyaluronidase isozymes from human urine. FEBS Lett 1997; 417:307–310.
5. Cherr GN, Yudin AI, Overstreet JW. The dual functions of GPI-anchored PH-20: hyaluronidase and intracellular signaling. Matrix Biol 2001; 20:515–25.
6. Sabeur K, Cherr GN, Yudin AI, Overstreet JW. Hyaluronic acid enhances induction of the acrosome reaction of human sperm through interaction with the PH-20 protein. Zygote 1998; 6:103–111.
7. Jones MH, Davey PM, Aplin H, Affara NA. Expression analysis, genomic structure, and mapping to 7q31 of the human sperm adhesion molecule gene *SPAM1*. Genomics 1995; 29:796–800.
8. Phelps B, Myles DG. The guinea pig sperm plasma membrane protein, PH-20, reaches the surface via two transport pathways and becomes localized to a domain after an initial uniform distribution. Dev Biol 1987; 123:63–72.
9. Jones R, Ma A, Hou ST, Shalgi R, Hall L. Testicular biosynthesis and epididymal endoproteolytic processing of rat sperm surface antigen 2B1. J Cell Sci 1996; 109:2561–2570.
10. Zheng Y, Martin-DeLeon PA. The mouse *Spam1* gene: RNA expression pattern and lower steady-state level associated with the Rb(6.16) translocation. Mol Reprod Dev 1997; 46:252–257.
11. Deng X, He Y, Martin-DeLeon PA. Mouse Spam1 (PH-20): evidence for its expression in the epididymis and for a new category of spermatogenic-expressed genes. J Androl 2000; 21:822–832.

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12. Zhang H, Martin-DeLeon PA. Mouse epididymal Spam1 (PH-20) is released in vivo and in vitro, and *Spam1* is differentially regulated in testis and epididymis. Biol Reprod 2001; 65:1586–1593.
13. Zhang H, Martin-DeLeon PA. Mouse epididymal Spam1 (PH-20) is released in the luminal fluid with its lipid anchor. J Androl 2003; 24: 51–58.
14. Frenette G, Lessard C, Sullivan R. Selected proteins of ''protasome-like particles'' from epididymal cauda fluid are transferred to epididymal caput spermatozoa in bull. Biol Reprod 2002; 67:308–313.
15. Hermo L, Badran H, Andonian S. The structural organization and functions of the epithelium of the vas deferens. In: Robaire E, Hinton BT (eds.), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer/Plenum; 2002:233–250.
16. Ekhlasi-Hundrieser M, Sinowatz F, De Wilkes IG, Waberski D, Topfer-Petersen E. Expression of spermadhesin genes in porcine male and female reproductive tracts. Mol Reprod Dev 2002; 61:32–41.
17. Guntenhoner MW, Pogrel MA, Stern R. A substrate gel assay for hyaluronidase activity. Matrix 1992; 12:388–396.
18. Csoka AB, Scherer SW, Stern R. Expression analysis of six paralogous human hyaluronidase genes clustered on chromosomes 3p21 and 7q31. Genomics 1999; 60:356–361.
19. Sassone-Corsi P. Unique chromatin remodeling and transcriptional regulation in spermatogenesis. Science 2002; 296:2176–2178.
20. Zheng Y, Martin-DeLeon PA. Characterization of the genomic structure of the murine *Spam1* gene and its promoter: evidence for transcriptional regulation by a cAMP-responsive element. Mol Reprod Dev 1999; 54:8–16.
21. Kashiwabara S, Noguchi J, Zhuang T, Ohmura K, Honha A, Sugiura S, Miyamoto K, Takahashi S, Inoue K, Ogura A, Baba T. Regulation of spermatogenesis by testis-specific, cytoplasmic poly(A) polymerase TPAP. Science 2002; 298:1999–2002.
22. Zheng Y, Deng X, Martin-DeLeon, PA. Lack of sharing of Spam1 (PH-20) among mouse spermatids and transmission ratio distortion. Biol Reprod 2001; 64:1730–1738.
23. Thaler CD, Cardullo RA. Biochemical characterization of a glycophosphatidyl-inositol-linked hyaluronidase on mouse sperm. Biochemistry 1995; 34:7788–7795.
24. Deng X, Czymmek K, Martin-DeLeon PA. Biochemical maturation of Spam1 (PH-20) during epididymal transit of mouse sperm involves modifications of *N*-linked oligosaccharides. Mol Reprod Dev 1999; 52:196–206.
25. Rutllant J, Meyers SA. Post-translational processing of PH-20 during epididymal sperm maturation in the horse. Biol Reprod 2001; 65: 1324–1331.
26. Kobata A. Structures and functions of the sugar chains of glycoproteins. Eur J Biochem 1992; 209:483–501.
27. Zanveld JD, DeJong CJ. Mammalian sperm acrosomal enzymes and the acrosome reaction. In: Dundar BS, O'Rand MG (eds.), Comparative Overview of Mammalian Fertilization. New York: Plenum; 1991:63–79.
28. Yang B, Yang B, Savani RC, Turley EA. Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44 and link protein. EMBO J 1994; 13:286–296.
29. Kobayashi H, Terao T. Hyaluronic acid-specific regulation of cytokines by human uterine fibroblasts. Am J Physiol 1997; 273:C1151–C1159.

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