Murine SPAM1 is secreted by the estrous uterus and oviduct in a form that can bind to sperm during capacitation: acquisition enhances hyaluronic acid-binding ability and cumulus dispersal efficiency

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Abstract

Sperm uptake of epididymal sperm adhesion molecule 1 (SPAM1) in vitro has recently been shown to be a marker of sperm maturation, since acquisition of this surface hyaluronidase increases cumulus dispersal efficiency. Here, we demonstrate that this glycosyl phosphatidylinositol-linked sperm antigen, previously shown to be expressed during estrous in the female reproductive tract, is secreted in the uterine and oviductal fluids (ULF and OF respectively) in a 67 kDa form, which can bind to sperm. We show that it can be acquired by caudal sperm from Spam1 null, Spam1-deficient mutant, and wild-type (WT) mice in vitro during incubation in ULF or OF at 37 °C, as detected by immunocytochemistry and flow cytometry. SPAM1 binding after ULF incubation was localized predominantly to the acrosome and the mid-piece of the flagella of Spam1 null sperm in a pattern identical to that of WT sperm. After ULF incubation, WT sperm demonstrated a significantly (P<0.001) enhanced hyaluronic acid-binding ability, and the involvement of SPAM1 in this activity was shown by a significant (P<0.001) decrease in binding when sperm were exposed to SPAM1 antiserum-inhibited ULF. Importantly, when Spam1 null sperm were exposed to ULF with SPAM1 accessible (in the presence of pre-immune serum) or inaccessible (in the presence of SPAM1 antiserum) for uptake, there was a significant difference in cumulus dispersal efficiency. Taken together, these results suggest that in the sperm surface remodeling that occurs prior to and during capacitation, the fertilizing competence of sperm is increased via acquisition of SPAM1, and likely other hyaluronidases, from the female tract.

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Introduction

It is known that in mice there are several reproductive hyaluronidas (Kim et al. 2005, Zhang et al. 2005) of which sperm adhesion molecule 1 (SPAM1), encoded by Spam1 on chromosome 6A2 (Deng et al. 1997), is the most well characterized (Lin et al. 1994, Thaler & Cardullo 1995, Deng et al. 1999). SPAM1, which is highly conserved (Lathrop et al. 1990), has been implicated in at least three major aspects of mammalian fertilization: (a) enzymatic degradation of HA and thereby assisting in the penetration of sperm through the cumulus layer surrounding the zona pellucida (Lin et al. 1994, Hunnicutt et al. 1996, Cherr et al. 2001), (b) zona pellucida binding (Myles & Primakoff 1997, Cherr et al. 2001), and (c) along with zona pellucida proteins, induction of acrosome reaction (Vandevoort et al. 1997, Sabeur et al. 1998, Cherr et al. 2001, Vines et al. 2001). This glycosyl phosphatidylinositol (GPI)-linked protein is expressed in the testes (Myles & Primakoff 1997, Zheng & Martin-DeLeon 1997, Seaton et al. 2000), and epididymis (Martin-DeLeon 2006), as well as in accessory organs such as the vas deferens, seminal vesicles, and prostate (Zhang et al. 2004, Martin-DeLeon 2006). It has also been shown to be secreted in vivo and in vitro with the latter occurring in the media of cultured murine epididymal epithelial cells (Zhang & Martin-DeLeon 2001).

Both the quantity and localization pattern of SPAM1 on sperm are altered as they traverse the epididymis: more SPAM1 is present on caudal sperm than on those from the caput (Deng et al. 1999, Rutllant & Meyers 2001, Morin et al. 2005, Martin-DeLeon 2006) and its distribution changes from being uniform to localized, predominantly on the plasma membrane of the anterior head (Deng et al. 1999). The quantitative change suggests that sperm acquire SPAM1 during epididymal transit. Supporting this idea are the following findings in rats: (1) after efferent duct ligation, SPAM1 accumulates in the epididymal lumen in the absence of sperm, (2) in unligated animals, caudal sperm that are positioned adjacent to the luminal epithelium stain...
more immunopositively for SPAM1 than those in the interior of the lumen, and (3) SPAM1 isoforms on sperm and the epididymal regions where they reside are the same and differ from those in the testes (Zhang et al. 2004).

Recently, our laboratory has shown that caudal mouse sperm can acquire SPAM1 in vitro from the epididymal luminal fluid (ELF) and that this uptake increases sperm fertilizing competence (Chen et al. 2006). We have also demonstrated that, similar to its expression in the male reproductive tract, SPAM1 is expressed in the glandular and surface epithelia of the female tract (Zhang & Martin-DeLeon 2003b). Expression in the female tract is highest during proestrus and estrous and is region dependent, decreasing from the vagina to the oviduct (Zhang & Martin-DeLeon 2003b). Importantly, SPAM1 in the female was shown to have hyaluronidase activity only at neutral pH, similar to epididymal SPAM1 and unlike SPAM1 found on caudal sperm for which this activity occurs at both neutral and acidic pH (Cherr et al. 2001, Zhang & Martin-DeLeon 2003b).

The purpose of this investigation was to determine whether murine SPAM1 is secreted into the lumen of the uterus and/or oviduct and, if so, whether it is a unique or common isoform. We also sought to determine whether SPAM1 can be transferred to the plasma membrane of caudal sperm via incubation in ULF or OF. Because SPAM1 mRNA and protein concentration as well as hyaluronidase activity are significantly lower in the oviduct than in the uterus (Zhang & Martin-DeLeon 2003b), we focus on the acquisition of SPAM1 from ULF with regard to its effect on sperm function.

**Results**

**SPAM1 Western blot analysis**

Western blotting shows that SPAM1 is present in murine OF and ULF during estrous (Fig. 1). The specificity of the SPAM1 antiserum used has been previously demonstrated using peptide inhibition (Deng et al. 2000, Zhang & Martin-DeLeon 2001, 2003a). The detected SPAM1 protein has a major band with a MW of ~67 kDa similar to that found in the uterine and oviductal tissues (Zhang & Martin-DeLeon 2003b) as well as ELF and testis extract (Zhang & Martin-DeLeon 2001, 2003a), used as positive controls. The minor bands found in OF are potentially representative of different states of glycosylation.

**SPAM1 immunocytochemistry**

To determine whether SPAM1 secreted by the female tract can be acquired by sperm, caudal sperm from wild-type (WT) and Spam1 null mice were incubated in 2% BSA, Spam1 null ULF or WT ULF from estrous females at 37 °C for 1.5 h. Post-incubation, fluorescent images resulting from immunocytochemistry revealed that SPAM1 secreted into ULF can be taken up by Spam1 null sperm (Fig. 2D). The localization patterns of SPAM1 post-acquisition mirror that of mature WT sperm (Fig. 2A) incubated under capacitation conditions, with SPAM1 displayed in a punctate manner on the plasma membrane of the anterior head, as well as the midpiece of the tail. This pattern of localization is in contrast with immature caput sperm where the protein is uniformly distributed on the head and localized only to the principal piece of the tail (Deng et al. 1999). Several dozen sperm were imaged per sample; a selection of photographs representative of typical results is shown in Fig. 2(A–D). It should be noted that not all Spam1 null caudal sperm incubated in ULF acquired comparable amounts of SPAM1; some sperm acquired more or less than others. However, sperm that demonstrated SPAM1 acquisition from ULF shared the same localization pattern, one that is typical of SPAM1 found on mature WT sperm incubated under capacitation conditions.

**Flow cytometric analysis of SPAM1**

Physiologically, mature caudal sperm with pre-existing SPAM1 would be exposed to SPAM1 in ULF. Therefore, to investigate whether sperm with endogenous SPAM1 are able to pick up exogenous SPAM1, we incubated caudal sperm from Rb(6.16)24Lub in PBS (carrier control) or ULF from estrous female mice under the aforementioned conditions. Caudal sperm from WT mice were incubated in PBS (carrier control), ULF, or OF. Sperm were then processed for ICC prior to being subjected to flow cytometric analysis to quantify uptake. Background level of autofluorescence was estimated...
using samples processed in the absence of primary antiserum (data not shown). Samples incubated in PBS were used to determine basal or endogenous SPAM1 levels prior to uptake and the difference between OF or ULF and PBS was deemed to be due to acquisition of exogenous SPAM1. Figure 2E reveals that Rh(6.16)24Lub acquire exogenous SPAM1 from ULF and Fig. 2F and G demonstrate that WT sperm acquire SPAM1 from both ULF and OF as demonstrated by increases in peak fluorescence intensities over control levels. Red blood cells (RBCs), on the other hand, were not able to acquire exogenous SPAM1 from ULF, as demonstrated by a lack of increase in peak fluorescence intensity over control levels (Fig. 2H).

**Hyaluronic acid-binding assay**

A total of 2284 WT sperm were analyzed for their ability to bind HA as per manufacturer’s instructions (see Table 1). Sperm exposed to ULF/HTF with pre-immune serum (PIS) showed 89.72% binding when compared with the 83.10% seen from those exposed to HTF alone. \( \chi^2 \) analysis demonstrated a highly significant difference \( (\chi^2 = 14.03; P < 0.001) \) between these datasets, using Yate’s correction in a 2x2 contingency table. Sperm exposed to ULF/HTF in the presence of the SPAM1 antiserum showed 53.04% bound, a drastic decrease in HA binding, which is highly significantly different from those in either the HTF \( (\chi^2 = 172.2, P < 0.001) \) or ULF/HTF PIS \( (\chi^2 = 209.5; P < 0.001) \).

**Cumulus dispersal assay**

To determine whether SPAM1 acquisition increases sperm fertilizing competence, 50 000 Spam1 null sperm were introduced to oocytes from superovulated females after exposure to ULF under conditions where SPAM1 was available for (Fig. 3C) and blocked from (Fig. 3B) binding. WT sperm incubated in ULF were used as a positive control (Fig. 3A). The progression of oocyte dispersal from stage 1 through stage 4 is seen in 10% intervals on the graphs. A total of 302 oocytes were
analyzed as follows: WT sperm in ULF, 77; \textit{Spam1} null sperm in ULF + Ab, 98; \textit{Spam1} null sperm + PIS, 127.

Eggs incubated with WT sperm all progressed past stage 1 by 150 min. In contrast, eggs incubated with null sperm exposed to WT ULF did not all progress past stage 1 until 240 (in the absence of SPAM1 antiserum) and 270 min (in the presence of SPAM1 antiserum). Progression from stage 2 into stages 3 and 4 was also hindered by the addition of the SPAM1 antiserum; by the end of the 5-h analysis, \%40\% of the oocytes remained in stage 2 in the

<table>
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<tr>
<th>Treatment</th>
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<th>Total # (n)</th>
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<td>Raw data</td>
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<tr>
<td>ULF/HTF + PIS*</td>
<td>576</td>
<td>66</td>
<td>642</td>
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<td>HTF alone*</td>
<td>846</td>
<td>172</td>
<td>1018</td>
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<tr>
<td>ULF/HTF + SPAM1 Ab</td>
<td>331</td>
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Statistical analysis

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\begin{align*}
\chi^2 = 14.03 & \quad P < 0.001 \\
\chi^2 = 209.5 & \quad P < 0.001 \\
\chi^2 = 172.2 & \quad P < 0.001
\end{align*}
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Sperm exposed to HTF/ULF in the presence of PIS showed an 89.72\% binding efficiency of HA, when compared with 83.1\% seen for sperm exposed to HTF alone*. This difference is significant as determined by \(\chi^2\) analysis \((P<0.001)\). When the 89.72\% binding efficiency seen for HTF/ULF in the presence of pre-immune serum was compared with the 53.04\% seen for HTF/ULF in the presence of a SPAM1 antiserum, the difference was also determined to be significant by \(\chi^2\) analysis \((P<0.001)\), and thus indicates that the increase in HA-binding efficiency seen post-HTF/ULF incubation is at least partially due to SPAM1 acquisition from the ULF.

Figure 3 (A–C) In vitro cumulus dispersion efficiency of sperm capacitated under various conditions, as measured by the percentage of eggs in each of four stages of cumulus dispersal over 5 h. Each line on the graphs represents a 10\% interval. WT ULF was used for capacitation of WT sperm (A) and \textit{Spam1} null sperm in the presence of SPAM1 antiserum (B) or pre-immune serum (C). (D) Data in A–C with respect to eggs in stage 4 at 5 h in all treatments are summarized. There is a highly significance difference for \textit{Spam1} null sperm in WT ULF + PIS versus WT ULF + SPAM1 Ab; \(P=2.2 \times 10^{-14}\).
presence of SPAM1 antiserum, while <10% remained in stage 2 in the absence of the SPAM1 antiserum, when compared with 0% in the positive control.

When the percentage of eggs that reached stage 4 of cumulus dispersal at the end of 5 h in all groups was compared (Fig. 3D), Spami null sperm exposed to ULF in the absence of SPAM1 antiserum but the presence of PIS performed highly significantly better than their negative control counterpart incubated in ULF containing SPAM1 antiserum (P=2.2×10^{-14}). Spami null sperm in the presence of PIS appeared to have performed less efficiently than those reported by Baba et al. (2002) where the dispersal rate was 60% of WT sperm for eggs in stage 4 by the end of the study. This may be due to differences in experimental design, including sperm concentration and period of incubation.

Discussion

Our results have demonstrated that the murine uterus releases SPAM1, a GPI-linked hyaluronidase, into the lumen during estrous. Thus, uterine SPAM1, like epididymal SPAM1 (Zhang & Martin-DeLeon 2001, 2003a), is a secretory protein. In the estrous uterus and oviductal fluids, the protein was shown to have a MW of ~67 kDa, similar to that found in uterine and oviductal tissues, as well as testes and epididymal tissues (Martin-DeLeon 2006). The 67 kDa protein is also found in caudal sperm, before it is proteolytically cleaved and deglycosylated, decreasing its MW to 53 kDa (Deng et al. 1999). This finding for uterine and oviductal SPAM1 suggests that SPAM1 is also likely to be secreted in the vagina, where the protein is also expressed in the luminal epithelium (Zhang & Martin-DeLeon 2003b).

Acquisition of uterine SPAM1 by caudal sperm following ULF and/or OF incubations in vitro was demonstrated both qualitatively (ULF) and quantitatively (ULF and OF) as seen in Fig. 2(E–G). These findings suggest that uterine and oviductal SPAM1 may be acquired in vivo during sperm capacitation. In vivo acquisition of proteins from the female tract has been previously demonstrated to be associated with increasing fertilizing competence. For example, it has been shown to occur for bovine (King et al. 1994) and hamster oviductin (Boatman & Magnoni 1995, Kan & Esperanzate 2006), which are similar to SPAM1 in being cyclically expressed oviductal glycoproteins known to play a role in capacitation and fertilization. Interestingly, like SPAM1, oviductin preferentially interacts with sperm on the region of the plasma membrane overlying the acrosomal cap (Kan & Esperanzate 2006). However, it should be noted that the type of interaction by which oviductin is attached to the membrane is as yet unclear, as is the specific role it plays in hamster capacitation or fertilization (Kan & Esperanzate 2006).

The demonstration that uterine SPAM1 is able to bind to the sperm plasma membrane suggests that it is secreted with an intact GPI anchor. This would be similar to epididymal SPAM1 that has been shown to be transferred to the sperm plasma membrane in vitro (Chen et al. 2006) and has a GPI anchor on the vesicular component of the ELF (Zhang & Martin-DeLeon 2003a). Our study therefore suggests that SPAM1, like other GPI-linked proteins, may be involved in the post-testicular remodeling of the sperm surface that occurs not only during their maturation and storage in the cauda, but also during capacitation (Kirchoff et al. 1997). However, while alterations in the distribution and location of GPI-linked proteins have been known to occur during capacitation (Kirchoff et al. 1997), to our knowledge, this may be the first reported GPI-linked protein with a known function transferred to the sperm surface from the uterine and oviductal fluids.

SPAM1 acquisition was more pronounced in the Rb(6.16) 24Lub sperm, which have 70% of WT levels of SPAM1 (Zheng et al. 2001), than in the WT sperm under similar conditions and protein concentrations. This suggests that sperm with less endogenous SPAM1 are more capable of acquiring exogenous SPAM1, and that WT sperm are perhaps closer to a saturation level expected to be reached prior to capacitation. Consequently, the in vitro binding of uterine SPAM1 demonstrated in this study suggests that binding or uptake occurs physiologically in the female tract after ejaculation, augmenting testicular, and/or epididymal SPAM1. The fact that caudal sperm have not reached maximal saturation increases the plausibility that in vivo ejaculated sperm can acquire SPAM1 from ULF and OF during capacitation.

What might be the significance of incremental acquisition of SPAM1 in the male and female reproductive tracts? Both murine and human SPAM1 have been implicated to be involved in the acrosome reaction (Sabour et al. 1998, Morales et al. 2004), one of the final events sperm undergo after primary zona pellucida binding and just prior to fertilization. If maximal levels of SPAM1 are reached prematurely, sperm might acrosome react prior to reaching the cumulus matrix of the egg. Thus, by incrementally adding SPAM1 to sperm during successive residence in the epididymis, uterus, and oviduct, it is possible that the levels required for the acrosome reaction are attained only at the appropriate time, after sperm traverse the uterus and arrive in the ampulla where fertilization occurs.

Thus far, in vivo cell-to-cell transfer of GPI-linked proteins is best known to occur for sperm and erythrocytes, cell types in which biosynthetic activity is limited or absent. In this study, uptake was shown to be cell-type specific since RBCs did not bind uterine SPAM1. Because RBCs are known to carry GPI-linked proteins (Civenni et al. 1998) acquired from the blood (Kooyman et al. 1995, Sloand et al. 1998), the lack of
SPAM1 acquisition in RBCs suggests that sperm may have specific lipid raft-associated microdomains (Sleight et al. 2005) within the plasma membrane for SPAM1 binding or adsorption, or alternatively, that there may be specific sperm receptors that mediate the binding. Interestingly, the localization of SPAM1 on WT sperm (Fig. 2A) parallels that of caveolin-1 reported by Travis et al. (2001) and both are known to be associated with lipid raft microdomains (light buoyant density fractions; Sleight et al. 2005).

In its native location on the sperm head, SPAM1 acquired from ULF functions physiologically, as demonstrated by the significantly enhanced HA-binding activity of WT sperm, which induces the acrosome reaction pathway (Sabeur et al. 1998, Morales et al. 2004), as well as inhibition of cumulus dispersal by Spam1 null sperm when SPAM1 acquired from ULF was blocked. The latter finding does not negate the involvement of other hyaluronidases present in the mouse, as the addition of the SPAM1 antiserum is likely to incur steric hindrance, which may inhibit the activity of other proteins in the same vicinity as SPAM1. Cumulus-penetrating ability is dependent on neutral hyaluronidase activity (Cherr et al. 2001), which was shown for uterine, as well as vaginal and oviductal SPAM1 (Zhang & Martin-DeLeon 2003b).

Our study shows that SPAM1 from the female reproductive tract, like epididymal SPAM1, is involved in the remodeling of the sperm surface during their maturation. Remodeling of the sperm PM in the female tract is known to occur prior to capacitation when there is cholesterol efflux (see Sleight et al. 2005). The findings for this study suggest that selective uptake of GPI-linked proteins such as SPAM1 may assist in the stabilization of the sperm membrane during cholesterol efflux as well as significantly contribute to multiple essential steps in fertilization.

Materials and Methods

Animal care and use

The following studies conform to the guide for the Care and Use of Laboratory animals published by the National Institutes of Health (publication 85–23, revised 1985) and were approved by the Animal Care Committee at the University of Delaware. The sexually mature 3- to 6-month-old male and 4- to 6-week-old (Institute of Cancer Research, ICR) female mice used throughout these studies were obtained from Harlan Sprague Dawley, Indianapolis, IN, USA. Age-matched Rb(6.16)24Lub, Robertsonian translocation-bearing animals, were obtained from Jackson Lab (Bar Harbor, ME, USA). Due to Spam1 mutations, these animals have only 70% of the WT levels of sperm SPAM1 (Zheng et al. 2001).

Generation and confirmation of Spam1 null animals

Spam1−/− (null) mice, generated on the ICR background, were a generous gift from the laboratory of Dr Tadashi Baba, University of Tsukuba, Tsukuba Science City, Ibaraki, Japan. The genotype of the mice in our colony was confirmed via PCR (using the primers described in Baba et al. 2002) and Northern analysis of testicular RNA. Mice from the Spam1 null colony show no decrease in fertility, an effect potentially due to a compensatory mechanism provided by other murine hyaluronidases, namely Hyal5 and Hyalp1.

Reagents

All reagents were purchased from Sigma Chemical Co. unless otherwise specified. The rabbit anti-mouse SPAM1 antiserum used throughout these studies is a polyclonal anti-peptide (C NEKGMASRRKESSD in the C-terminus (no. 381–395)) custom made by Zymed, San Francisco, CA, USA (Deng et al. 2000).

Collection of sperm, ELF, and testis protein

Caudal epididymides were finely minced in normal PBS (pH 7.4, 37 °C) and sperm were allowed to swim into solution for 10 min. After sperm dispersion in the suspension, tissue fragments were separated by gravity settling. The suspension was then centrifuged at 500 g for 15 min in a 1.5 ml eppendorf tube to pellet the sperm while minimizing membrane damage. The resulting supernatant was further centrifuged at 16 100 g for 20 min in a 1.5 ml eppendorf tube to remove sperm and RBCs, creating the ELF used as a positive control in the Western blot analysis. Sperm pellets were washed twice by centrifugation and resuspended in PBS to completely remove all epididymal fluid. Testis proteins were obtained by homogenization of WT testis tissue (in PBS) using a mortar and pestle, followed by centrifugation of the tissue at 16 100 g for 20 min. The testis protein collected in the supernatant was used as an additional positive control in the Western blot analysis. All protein concentrations were obtained using a bicinchoninic acid assay (BCA kit, Pierce, Rockford, IL, USA).

Collection of uterine luminal fluid and oviductal fluid

ULF and OF were obtained from mice artificially induced into estrous via i.p. injections of 7.5 i.u. equine chorionic gonadotropin (eCG) and 7.5 i.u. human chorionic gonadotropin (hCG) spaced 48 h apart. Uteri were removed 13.5–14 h after hCG injections and flushed with either human tubal fluid (HTF; Chemicon International, Temecula, CA, USA), a known capacitation medium (Kito & Ohta 2005), or PBS. Oviducts were removed and minced in PBS. Protein concentrations were obtained using a bicinchoninic acid assay (BCA kit, Pierce, Rockford IL) after the luminal fluids (2–3 ml) were subjected to centrifugation at 3500 g for 10 min to pellet blood cells and excess tissue.

Collection of RBCs

Whole blood was collected from WT 6- to 8-week-old female mice and heparin sulfate (100 i.u./ml) was added to prevent clotting. Whole blood+heparin sulfate (2 ml) was added to
2 ml of 20% Ficoll (w/v in PBS). The suspension was centrifuged at 1000 g for 10 min. RBCs were collected from the bottom of the tube. Cell counts were calculated using a hemocytometer.

Western blot analysis

Western blotting was performed with the WesternBreeze Chemiluminescent Immunodetection Kit (Invitrogen), and all incubations were carried out according to the manufacturer’s instructions at room temperature (RT), with gentle shaking. Samples of ~20–40 µg ULF or OF protein were solubilized by SDS sample buffer and reduced by 100 mM dithiothreitol, subjected to 15% SDS-PAGE (Sambrook & Russell 2001) and transferred to a nitrocellulose membrane overnight (200 mA). BSA (2% w/v in PBS), ELF, and testes proteins were used as negative and positive controls respectively. The membrane was incubated in blocking solution (2% BSA in PBS) for 30 min. After decanting the blocking solution, the membrane was rinsed twice in 20 ml ddH₂O for 5 min and incubated in 10 ml rabbit anti-mouse SPAM1 antiserum (suspended in 2% BSA blocking solution, 1:1000 dilution) for 1 h. After washing in Ab wash solution provided with the kit, it was incubated in 10 ml anti-rabbit IgG secondary antibody solution provided with the kit for 30 min, Ab washed once more, and rinsed twice in 20 ml ddH₂O. SPAM1 immunodetection was performed using the Chemiluminescent Substrate, also provided with the kit.

Immunocytochemistry (ICC) of SPAM1

For a qualitative analysis of SPAM1 uptake and detection of its localization pattern on the sperm post-incubation, ICC was performed. Approximately 1 × 10⁶ sperm, obtained from the caudal epididymis of Spam1 null mice, were exposed to 2–3 ml Spam1 null or WT ULF (both in PBS, protein concentration 1–1.5 mg/ml) or PBS containing 2% BSA for 1 h at 37 °C, the latter serving as a negative control. WT sperm incubated in WT ULF were used as a positive control. Post-incubation, sperm were pelleted (500 g, 25 min) and washed twice in PBS to remove ULF. They were then fixed in 1.5% paraformaldehyde (16% paraformaldehyde stock solution in ddH₂O, diluted 1:10.5 v/v in PBS) for 1 h at RT or overnight at 4 °C. Then they were rinsed twice, resuspended in PBS+2% BSA block for 30 min at RT, before treatment with SPAM1 antiserum (suspended in 2% BSA block, 1:400 dilution) for 1 h at RT. They were then rinsed thrice in PBS and resuspended in FITC-conjugated goat anti-rabbit IgG secondary antibody (1:400 dilution) for 30 min in the dark. After washing thrice in PBS, sperm were resuspended in 20–40 µl PBS and 10–20 µl of each sample were spread on microscope slides and allowed to dry in the dark. Dried samples were stained with 0.1% in 50% glycerol in PBS with 1.5 µg/ml of 4′,6-diamidino-2-phenylindole (DAPI) sealed under a coverslip with clear nail polish and imaged using a Zeiss Axioskop. (Carl Zeiss, Oberkochen, Germany).

Flow cytometric analysis of SPAM1

To quantify SPAM1 uptake, caudal sperm from Rb(6.16) and WT mice were incubated in WT estrous ULF or OF under the same conditions described for Spam1 null sperm, except that the sample size ranged from 250 000 to 500 000 sperm per treatment. SPAM1 immunostaining was also identical to that described for Spam1 null sperm. Control samples were incubated in PBS and test samples in SPAM1 antiserum (1:400) for 1 h at RT. They were washed thrice in PBS, incubated in FITC-labeled goat anti-rabbit IgG (1:400) for 30 min, in the dark at RT, and washed thrice in PBS. To determine cell-type specificity of uptake, RBCs were incubated in WT ULF or PBS, and used as a negative control. Samples were then analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) flow cytometer that uses an argon laser at 488 nm with detectors for fluorescein and a Cell Quest software package (Becton Dickinson). For each treatment, 50 000 cells were analyzed via flow cytometry.

Hyaluronic acid-binding assay

WT sperm (pooled from three males) were exposed for 2 h (37 °C) to either WT estrous ULF flushed with HTF (ULF/HTF, total protein concentration ~3 mg/ml) in the presence of SPAM1 antiserum (1:400), rabbit PIS (1:400), or HTF alone. Post-incubation, 20 µl of each sperm sample were placed onto HA-coated slides (Biocoat Inc., Fort Washington, PA, USA), covered with a gridded coverslip, and permitted to bind to the HA substrate for 10–15 min at RT. Sperm were analyzed for HA-binding ability via phase microscopy. Sperm showing progressive motility were considered unbound, while sperm exhibiting motility without progressive movement were considered bound and non-motile sperm were not counted. The percentage of HA-bound sperm was calculated by dividing the total number of bound sperm by the total number of motile sperm (bound sperm plus unbound sperm) × 100. Approximately, 200–400 sperm were analyzed per sample, and 600–1000 sperm were analyzed per treatment. Data were combined from experiments run in triplicate.

Cumulus dispersal assay

Briefly, four 6-week-old ICR WT females were superovulated for each experiment, via injections (7.5 i.u.) of eCG and hCG, spaced 46–48 h apart. Females were killed 13.5–14 h after the hCG injection, their eggs collected, and their uteri flushed with HTF. The oocytes were placed in dishes containing 0.5 ml pre-warmed HTF, under a thin layer of mineral oil, and incubated at 37 °C in 90% N₂, 5% CO₂, and 5% O₂. Caudal sperm were collected from the epididymides of both a donor WT and a Spam1 null male, both ~6 months old. Sperm were allowed to swim out of cuts made in the caudal epididymides in HTF for 10 min at 37 °C. Then ~1 million caudal WT sperm were incubated under capacitation conditions in WT ULF/HTF and ~1 million Spam1 null sperm were incubated under capacitation conditions in ULF/HTF in the presence SPAM1 antiserum (Ab) or PIS for 1 h at 37 °C.
Therefore, the three groups in this study were: (1) WT sperm incubated in ULF, serving as a positive control, (2) Spam1 null sperm incubated in ULF + SPAM1 antiserum (Ab; 1:400 dilution), serving as a negative control, and (3) Spam1 null sperm incubated in ULF + PIS (1:400 dilution), the experimental group. Post-capacitation, ~50 000 sperm were introduced to each dish of eggs. The eggs were monitored every 30 min for 5 h, and the percentage of eggs in each stage of cumulus dispersal was recorded and graphed against time (in 30 min increments). The stages of cumulus dispersal were assessed similar to Chen et al. (2006) and Miller et al. (2007) using the following criteria: stage 1 eggs are surrounded by tightly packed cumulus cells such that they occlude the view of the oocytes. Stage 2 eggs retain most of their cumulus mass, however, enough cumulus cells are dispersed so that the oocytes are partially visible. Eggs in stage 3 have shed most but not all of the cumulus mass and their zona pellucida has become visible. Eggs that are essentially cumulus free are classified as stage 4. Experiments were run in triplicate to validate results.

Statistical analyses
The results obtained in the HA-binding assay were subjected to $\chi^2$ analysis, using Yates’s correction. Fisher’s exact test was used to determine statistical significance for the cumulus dispersal assay.

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