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Mouse Epididymal Spam1 (PH-20) Is Released in the Luminal Fluid With its Lipid Anchor

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ABSTRACT: Previously we demonstrated that the murine sperm adhesion molecule 1 (Spam1 or PH-20) is synthesized by the epididymal epithelium, preferentially in the distal region, and is released into the luminal fluid. We also showed that whereas testicular and epididymal Spam1 have hyaluronidase activity at neutral pH, they are under different transcriptional regulation. The aim of this study was to further compare characteristics of the two forms of this glycosyl-phosphatidylinositol-linked protein and their transcripts, and to determine whether secreted epididymal Spam1 is released with its lipid anchor. With GeneRacer amplification of the 3′ end of the complementary DNA we show that the poly(A) tails are significantly ($P < .05$) shorter in the epididymis than in the testis. Two-dimensional polyacrylamide gel electrophoresis with immunoblotting reveals one to three isoforms for epididymal Spam1 with the isoelectric point (pI) ranging from 7.3 to 9.0, and four isoforms ranging from 6.6 to 9.0 pI for testicular Spam1. Two isoforms with a pI ranging from 7.6 to 9.0 were observed for caudal sperm. Lectin blotting analysis shows that *Phaseolus vulgaris* erythroagglutinin, *Lycopersicon esculentum* lectin (LEL), and *Solanum tuberosum* lectin, which all bind to *N*-linked chains, recognize a 67 kd band in the epididymis and caudal sperm, but not in the testis. Treatment of the protein extracts with anti-Spam1 serum prior to blotting with LEL led to the disappearance of the banding, indicating Spam1 specificity of the staining. The lectin peanut agglutinin, which preferentially binds to *O*-linked side chains, recognizes a 67 kd band in all three cell types. Enzymatic deglycosylation studies confirmed the presence of an *O*-linked glycan in all three cell types. Ultracentrifugation of the luminal fluid reveals that epididymal Spam1 is secreted predominantly as insoluble particles, which when treated with phosphatidylinositol-specific phospholipase C or Triton X-100, reveal that the majority of epididymal Spam1 is released with its lipid anchor, a form in which it can bind to sperm.

Key words: GPI-anchor, insoluble particles, epididymosomes.
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The sperm adhesion molecule 1 (SPAM1 or PH-20) is a glycosyl-phosphatidylinositol (GPI)-linked sperm surface glycoprotein with multiple roles in mammalian fertilization. Along with its best known function as the sperm hyaluronidase, which is essential for cumulus penetration (Gmachl et al, 1993; Lin et al, 1994; Cherr et al, 2001), it is known to have zona pellucida-binding activity (Primakoff et al, 1985; Gmachl et al, 1993) and to be involved in the signaling associated with acrosomal exocytosis (Cherr et al, 2001). Recently we demonstrated that murine Spam1, which like that of other species (Phelps and Myles, 1987; Zheng and Martin-DeLeon, 1997), was believed to be testis-specific, is also synthesized in the epididymal epithelium, and is released in the luminal fluid (Deng et al, 2000; Zhang and Martin-DeLeon, 2001). Epididymal Spam1 (ES), like testicular Spam1 (TS), was shown to have hyaluronidase activity at neutral pH, but is under a different transcriptional regulation from that of TS (Deng et al, 2000; Zhang and Martin-DeLeon, 2001).

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Because secretory epididymal proteins are involved in the morphological, physiological, and biochemical changes that mammalian sperm undergo during epididymal transit when they acquire motility and fertilizing capability (Orgebin-Crist, 1967; Cooper, 1990; Kirchhoff et al, 1998), it is possible that ES may play a role in sperm maturation. This may occur if ES binds to sperm either as a unique isoform or for the purpose of enhancing Spam1 of testicular origin. The aim of this study was to further characterize ES by comparing it with TS, and to determine whether it is released with its lipid anchor intact, a form in which it can bind to sperm.

For the comparison we investigated both the protein and the transcript, where we focused on the 3′ untranslated region (UTR) and the poly(A) tails, which are known to play an important role in messenger RNA (mRNA) stability (Wickens et al, 1997). To study the protein we used two-dimensional gel electrophoresis and lectins to investigate the diversity of the associated oligosaccharides in the two tissues, as well as on sperm. Treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) or Triton X-100 was carried out to determine whether ES in the luminal fluid is associated with membrane particles and is secreted with its GPI anchor. Our results indicate that the mRNA transcripts are significantly shorter in the epididymis, and that TS and ES have

different *N*-linked glycosylation patterns with the latter showing glycans identical to those in caudal sperm. Our findings also reveal that ES is secreted with its lipid anchor, a form in which it can bind to sperm.

Materials and Methods

Animals and Reagents

Sexually mature ICR outbred male mice obtained by Harlan Sprague-Dawley, Inc (Indianapolis, Ind) were used in all studies. 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 Epididymis and Testis

Total RNAs from testes, and corpus and caudal epididymides were extracted using Tri-reagent according to the manufacturer's protocol. RNA samples were further treated with RNase-free DNase (Boehringer-Mannheim, Indianapolis, Ind; final concentration 5–10 U/mL) for 2–4 hours at 37°C, followed by phenol/chloroform extraction and ethanol precipitation.

Rapid Amplification of the 3′ Complementary DNA End

Amplification of the 3′ *Spam1* complementary DNA (cDNA) end was done using the GeneRacer Kit (Invitrogen, Carlsbad, Calif). Briefly, cDNA was generated using avian myeloblastosis virus reverse transcriptase and GeneRacer Oligo(dT) primer from 3 μg of total RNA. One microliter of diluted (twofold) cDNA was subjected to the polymerase chain reaction (PCR) to amplify the 3′ end using a forward gene-specific primer designed from the mouse *Spam1* cDNA sequence (nt 1896–1920; GenBank accession number U33958) and the GeneRacer 3′ primer provided with the kit. Nested PCR, using the same gene-specific primer and GeneRacer 3′ nested primer, was performed. After gel-purification, the PCR product was cloned into pCR 4-TOPO TA vector according to the manufacturer's instructions (Invitrogen). Several clones were isolated, plasmid DNA was prepared, and inserts were subjected to automated sequencing in our core facility.

Preparation of Protein Extracts From Sperm, Testis, Epididymal Tissues, and Luminal Fluids

Protein extracts from testis, caudal sperm, and epididymal tissues were prepared according to the methods described by Cherr et al (1996) and Deng et al (2000).

Sperm—To collect sperm, cauda epididymides were thoroughly minced and incubated in 2 mL of sperm suspension buffer (50 mM Tris, 20 mM EDTA, 1 mM *p*-hydroxy-mercurobenzenzoate, 5 mM *N*-ethylmaleimide, and 1 mM benzamidine pH 7.2) to disperse the sperm and allow them to swim out. The sperm suspension was centrifuged at 500 × *g* for 2 minutes to pellet the tissues, and sperm were collected by centrifuging at 1000 × *g* for 10 minutes. Protein extracts were prepared by lysing sperm with a solubilization buffer (62.5 mM Tris-HCl, 10% glycerol, 1% sodium dodecyl sulfate [SDS] pH 6.8) at 4°C at a final sperm concentration of 1×10^5 sperm/μL. The suspension was vigorously vortexed for 3 minutes and then centrifuged at 10 000 × *g* for 10 minutes, and the supernatant containing proteins was collected.

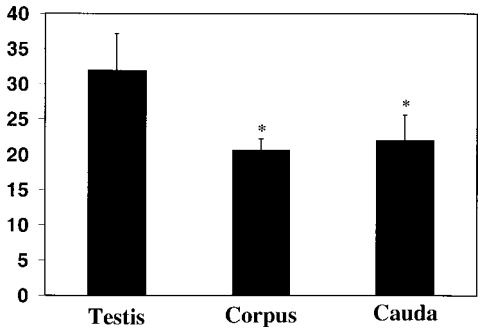

Figure. 1. Variation in the length of Poly(A) tails of *Spam1* mRNA populations expressed in different regions of the male reproductive system. Three colonies were picked and sequenced from each tissue (values are means ± SD). *Significantly different from testis value, $P < 0.05$.

Testicular and Epididymal Tissue—Testes were minced and the proteins were extracted with solubilization buffer. Minced epididymal tissues from which sperm were previously collected were washed a total of six times with Whittingham buffer containing 1 mM phenylmethylsulphonyl fluoride (PMSF) (centrifuged at 500 × *g* for 2 minutes) to remove adhering sperm until the sperm concentration in the final supernatant (5 mL) was less than 100 sperm/μL, and the proteins were extracted with the solubilization buffer. Tissues from this washing procedure were previously shown to harbor 10^3 sperm, which is 2 orders of magnitude lower then the 10^5 cells required to provide a band in Western analysis (Deng et al, 2000).

Luminal Fluids—Collection of epididymal luminal fluids was performed according to our previous procedure (Zhang and Martin-DeLeon, 2001) with some modifications. Briefly, the epididymides were isolated and divided into caput, corpus, and cauda. Each segment was minced into 2 mL of Whittingham buffer containing 1 mM PMSF and gently shaken to permit dispersal of the luminal contents. After the tissue pieces were allowed to settle, the upper fraction containing sperm and luminal fluid was centrifuged at 1000 × *g* for 3 minutes to remove cells. The supernatant obtained was then centrifuged at 3500 × *g* for 20 minutes at 4°C to remove cellular fragments. The proteins in the supernatant were precipitated with three volumes of acetone and recovered in sample buffer.

Two-Dimensional Polyacrylamide Gel Electrophoresis and Western Blot Analysis

First-dimensional electrophoresis was performed using the IPGphor Isoelectric Focusing System (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Briefly, protein samples were solubilized in a focusing solution containing 8 M urea, 2% CHAPS (w/v), 0.5% IPG Buffer type pH 3-10L (Amersham Pharmacia Biotech) and 18 mM dithiothreitol (DTT). Solubilized samples were then loaded on pH 3-10 linear gradient 7 cm IPG dry strips (Amersham Pharmacia Biotech) and isoelectric focusing was performed. Following this, the strips were subjected to 15% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membrane ac-

cording to standard protocols. Western blotting was performed with the WesternBreeze Chemiluminescent Immunodetection Kit (Invitrogen) according to the manufacturer's protocol. In brief, the membrane was blocked for 30 minutes at room temperature, then probed with rabbit antipeptide Spam1 antiserum (specific for Spam1; Deng et al, 2000), diluted 1:1000 in blocking solution. After washing, the membranes were incubated with anti-rabbit immunoglobulin G (IgG) biotin-conjugated (1:150 000 in blocking buffer). The protein was visualized by the chemiluminescence substrate provided with the kit.

Lectin Blotting

Reduced protein samples (heated at 99°C for 4 minutes in the presence of 100 mM DTT) were subjected to 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 3% w/v bovine serum albumin (BSA) in TTBS (50 mM Tris-HCl, 0.3 M NaCl, and 0.02% v/v Tween-20 pH 7.5) at room temperature overnight. The blots were then incubated for 2 hours separately with the following nine biotinylated lectins: concanavalin A (Con A), peanut agglutinin (PNA), *Pisum sativum* agglutinin (PSA), *Lens culinaris* agglutinin (LCA), *Phaseolus vulgaris* erythroagglutinin (PHA-E), Jacalin, *Griffonia simplicifolia* lectin I (GSL I), *Lycopersicon esculentum* lectin (LEL), and *Solanum tuberosum* lectin (STL) (Vector Laboratories, Burlingame, Calif) at a concentration of 1 μg/mL in TTBS containing 3% BSA and 10 mM Ca^{2+} and 10 mM Mn^{2+} at room temperature. After thorough washing with TTBS, membranes were incubated with alkaline phosphatase antibiotin antibodies (1:1000 in blocking buffer) (Vector Laboratories) for 1 hour. Positive staining was developed using the 1-Step NBT/BCIP (Pierce, Rockford, Ill). Preincubation of proteins extracted from caudal sperm and epididymal tissue with rabbit antipeptide Spam1 antiserum or preimmune serum was used to determine the identity of an N-linked band.

O-Linked Deglycosylation

O-Linked deglycosylation was performed as described by Anakwe et al (1991) with some modifications. In brief, protein extracts were incubated with 80 mU/mL of *O*-glycosidase for 20 hours at 37°C in 50 mM sodium phosphate supplemented with 1 mM PMSF pH 6.0. Control samples were incubated in the same conditions without *O*-glycosidase. The deglycosylated proteins were then used for Western blot analysis.

Preparation and PI-PLC Treatment of Insoluble Particles in Luminal Fluid

Insoluble particles were prepared from epididymal luminal fluid and treated with PI-PLC as described by Frenette and Sullivan (2001). In brief, epididymal luminal fluid was collected from 20 mice, subjected to 2 centrifugations as described above, and then ultracentrifuged at 120 000 × *g* for 2 hours. Pellets of insoluble particles were resuspended in 150 mM NaCl with or without 5 U/mL of PI-PLC for 15 minutes at 30°C, or treated with 1% Triton X-100 for 10 minutes at 4°C. The insoluble particles were then pelleted by ultracentrifugation at 120 000 × *g* for 2 hours. Both pellets and supernatants (from equivalent volumes of luminal fluid) were subjected to SDS-PAGE and immunoblotting analysis.

Results

Length of Poly(A) Tails of Epididymal and Testicular Spam1 Messenger RNA

A fragment of the 3′ UTR and the lengths of Poly(A) tails of *Spam1* mRNA in testis, and corpus and cauda epididymis were determined by 3′ rapid amplification of cDNA ends (RACE). Three colonies were picked and sequenced from each tissue. While the sequence of the 3′ UTRs preceding the poly(A) tails were identical in all samples, the poly(A) tails were shorter in the epididymis. They ranged from 19 to 22 residues (average 21) in the corpus and from 18 to 25 (average 22) in the cauda. However, in testis the tails ranged from 26 to 35 residues (average 32). Thus, whereas the two regions of the epididymis had poly(A) tails with similar lengths, the testis had tails that were significantly ($P < .05$) longer than those in the epididymis (Figure 1).

Analyses of Two-Dimensional PAGE and Immunoblotting Revealed Different Spam1 Isoforms in Epididymis, Testis, and Caudal Sperm

With two-dimensional PAGE and immunoblotting, one to three isoforms for epididymal Spam1 were observed in proteins from tissues as well as luminal fluid in all three regions. For all these, the isoelectric point (pI) ranged from 7.3 to 9.0. On the other hand, for TS there were four isoforms in a pI range of 6.6 to 9.0. Caudal sperm revealed two isoforms with the pI ranging from 7.6 to 9.0 (Figure 2).

Oligosaccharides of Spam1 in Epididymis, Testis, and Caudal Sperm

Information about oligosaccharides in the epididymis, testis, and caudal sperm was obtained by probing Western blotted proteins with lectins having known carbohydrate specificities. Of the 9 different lectins tested, PHA-E, LEL, and STL, which all bind to *N*-linked side chains, recognized a 67 kd molecular mass in the epididymis and caudal sperm, but not in testis (Figure 3). After preincubating proteins from the epididymis and caudal sperm with anti-Spam1 antiserum or preimmune serum, the LEL staining disappeared with anti-Spam1, but not with the control preimmune serum, indicating that Spam1 is responsible for the presence of the staining. Further, lectin blotting (using LEL and STL) of two-dimensional SDS-PAGE revealed a staining at 67 kd with a pI of 7.6 to 8.3 in epididymal tissue and luminal fluid (data not shown) where Spam1 protein was identified (Figure 2).

PNA, which preferentially binds to *O*-linked side chains, recognized the 67 kd molecular mass in both epididymis and testis as well as caudal sperm (Figure 3). On the other hand, Con A, GSL I, Jacalin, LCA, and PSA

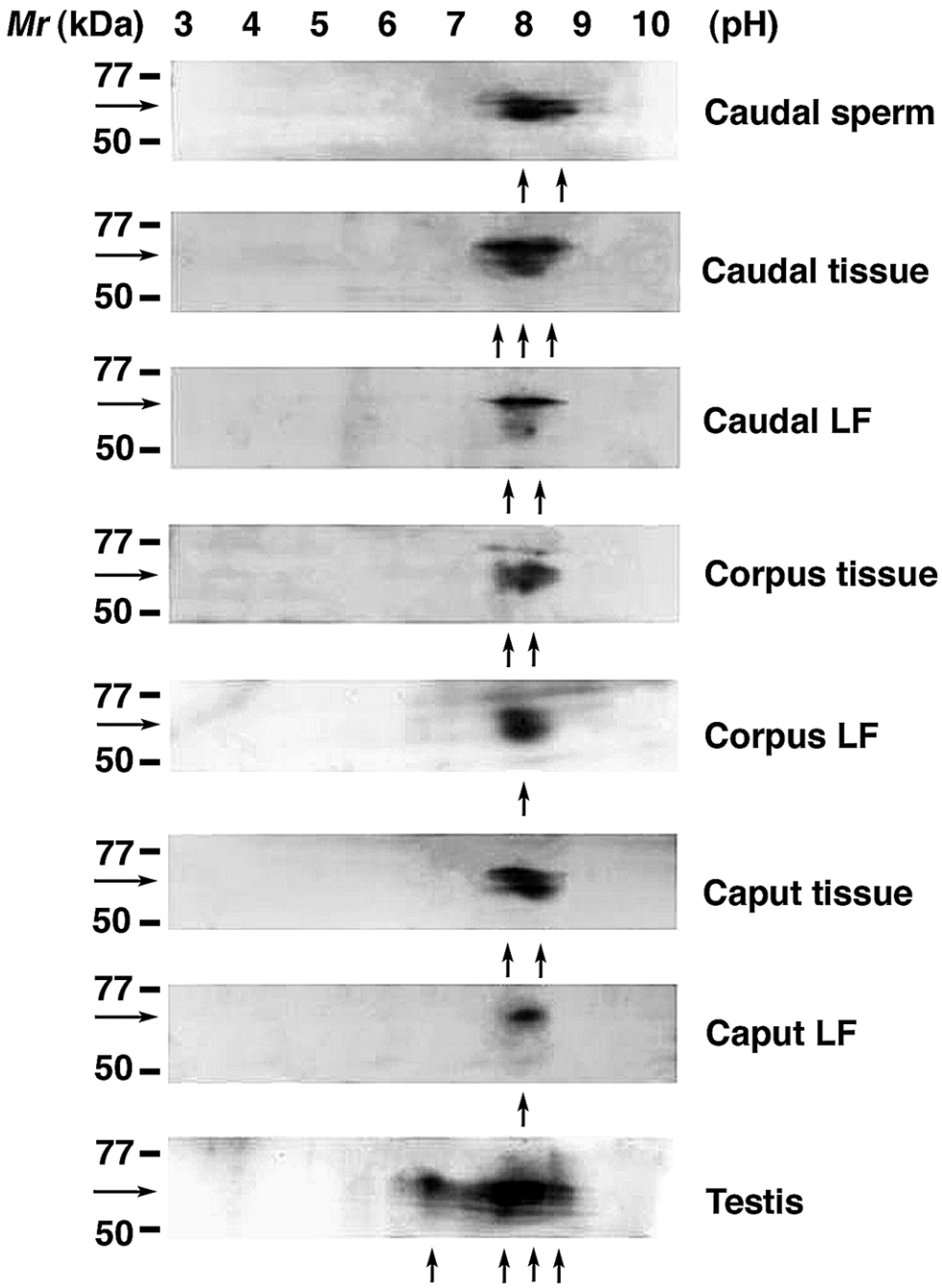

Figure 2. Western blot analysis of two-dimensional SDS-PAGE of Spam1 from testis, epididymis, and caudal sperm, revealing respectively, four testicular isoforms (pI range 6.6–9.0), one to three epididymal isoforms (pI range 7.3–9.0), and two isoforms (pI range 7.6–9.0) from caudal sperm. Only the region of the blot showing labeling is illustrated.

did not recognize the 67 kd molecular mass in any of the protein extracts (data not shown).

Enzymatic O-Linked Deglycosylation of Spam1 Suggests That the Protein Contains a Functional O-Linked Site

To date Spam1 has not been known to contain an *O*-linked sugar, although in a previous study we used computer analysis of the cDNA sequence to demonstrate the presence of a potential *O*-linked glycosylation site at position Thr379 (Deng et al, 1999). To determine whether the 67 kd protein that was positive for a PNA glycan in all samples (Figure 3) could be Spam1, *O*-linked deglycosylation was performed on extracts from testis, epididymis, and caudal sperm. After deglycosylation, proteins from all three regions show a 3 kd reduction in molecular

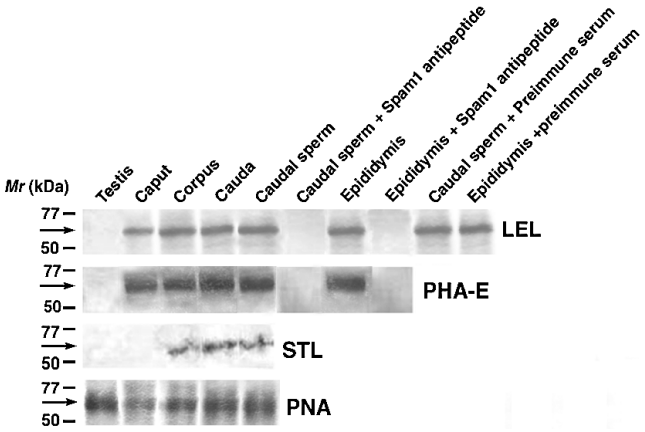

Figure 3. Lectin blots of proteins extracted from testis, epididymis, and caudal sperm with or without prior treatment with anti-Spam1 antibody.

weight (Figure 4), confirming that the potential *O*-linked site is functional. This suggests that the 67 kd protein that is positive for PNA in testis, epididymis, and sperm is likely to be Spam1.

ES Is Released Predominantly as Insoluble Particles in the Luminal Fluid and Contains its Lipid Anchor, as Revealed by Treatment With PI-PLC or Triton X-100

To determine whether released ES is soluble in the luminal fluid or associated with membranous particles, components of the luminal fluid were separated by ultracentrifugation and Spam1 antibody was used to detect the protein in the supernatant and the insoluble fractions. Using antipeptide Spam1 antibody, Spam1 in epididymal luminal fluid was predominantly associated with insoluble particles, and to a lesser extent with the supernatant (Figure 5, B and A, respectively). When the insoluble particles were treated with PI-PLC, which is known to cleave PI-anchored plasma membrane proteins from sperm (Phelps et al, 1988; Thaler and Cardullo, 1995; Legare et al, 1999), more than half (~60%) of Spam1 was released (Figure 5, E and F). Conversely, the same protocol applied without PI-PLC did not remove any Spam1 from the insoluble particles (Figure 5, C and D). Furthermore, 1% Triton X-100 was able to extract all the Spam1 from the insoluble particles (Figure 5, G and H), confirming that the protein is released in a membrane-bound form.

Discussion

Variation in the Length of Poly(A) Tails of Spam1 mRNA Populations in Testis and Epididymis

The *Spam1* cDNA (from testis) reported in the database (GenBank accession number U33958) contains a poly(A) tail of 29 residues, similar to the finding in this study of an average of 32 residues for testicular transcripts. Our

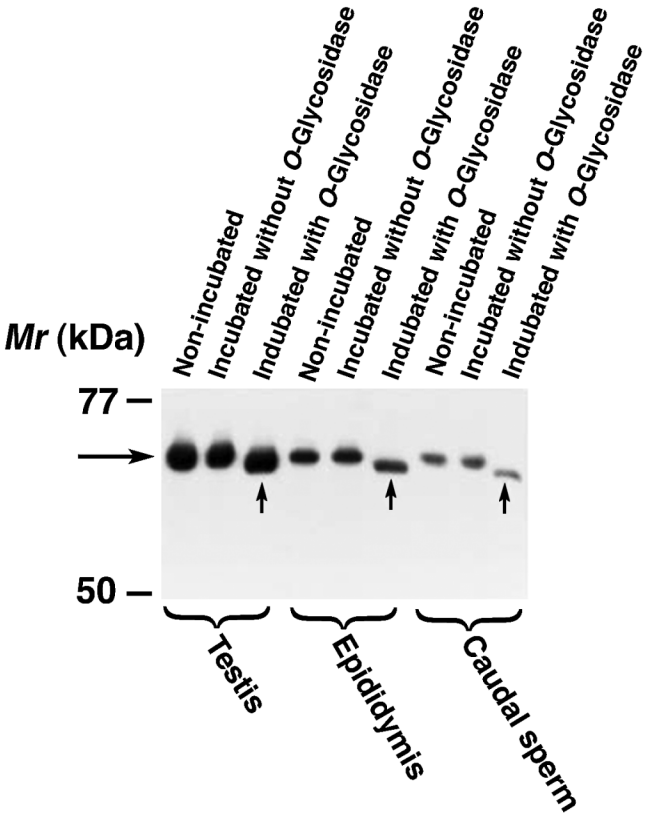

Figure 4. Western blot analysis of *O*-linked deglycosylated extracts from testis, epididymis, and caudal sperm. The three small arrows indicate the *O*-linked deglycosylated Spam1, which has a 3 kd reduction in molecular weight. Only the region of the blot showing labeling is illustrated.

results also show that the testicular transcripts, which are spermatid-derived (Zheng and Martin-DeLeon, 1997), are on average 10–11 residues longer—a significant difference—than those in the epididymis (Figure 1). It should be noted that spermatidal mRNAs with poly(A) tracts of 30–150 bases long are translationally active (Kleene, 1989), and so the length of the poly(A) tract observed for

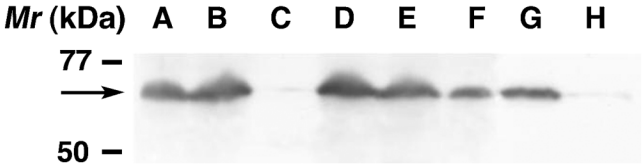

Figure 5. Immunodetection of Spam1 released from insoluble particles of epididymal luminal fluid after exposure to PI-PLC or Triton X-100. **(A** and **B)** Immunodetection in supernatant and pellet, respectively, from the initial ultracentrifugation (120 000 × *g* for 2 hours). In **(C** and **D)**, Spam1 was analyzed in the supernatant and pellet resulting from an aliquot of the pellet in **(B)** after treatment with buffer alone and secondary ultracentrifugation. **(E** and **F)** Immunodetection of supernatant and pellet resulting from an aliquot of the pellet in **(B)** after treatment with PI-PLC and secondary ultracentrifugation. In **(G** and **H)**, protein was analyzed in the supernatant and pellet resulting from an aliquot of the pellet in **(B)** after treatment with Triton X-100 and secondary ultracentrifugation. Only the region of the blot showing labeling is illustrated.

testicular *Spam1* transcripts would be consistent with translational activity.

Although a correlation between a specific number of residues in the poly(A) tract and translational activity has not been reported for epididymal mRNAs, it is known that androgens exert a positive effect on ''short'' (but not ''long'') poly(A) tails of CD52, a glycoprotein secreted by the epididymis and found on the surface of epididymal sperm (Pera et al, 1997). Thus it is possible that the maintenance of epididymal *Spam1* mRNAs with short tails might be regulated by androgens, which would not be the case for spermatidal mRNAs, because germ cells are without androgen receptors. The tissue difference in poly(A) tail length seen in this study may therefore well reflect regulation of expression (Curtis et al, 1995).

Oligosaccharides of Spam1 Show Tissue Differences in Epididymis and Testis, and Sperm and Epididymis Have Similar Glycan Structures

In the present study, Western blot analysis of two-dimensional SDS-PAGE was used to further identify biochemical characteristics of epididymal Spam1. Our results indicate that different patterns of isoforms of Spam1 exist in the testis and the epididymis. This correlates with the finding that Spam1 is synthesized independently in the testis and epididymis (Deng et al, 2000; Zhang and Martin-DeLeon, 2001), and suggests that its expression in the latter may not be redundant. There was more diversity in the testis which has four discrete isoelectric variants from 6.6 to 9.0 compared to the one-to-three isoforms in the epididymis spanning 7.3–9.0. The two isoforms with pI ranging from 7.6 to 9.0 observed in caudal sperm are within the range of those found in the testis and epididymis. This suggests that populations of Spam1 on sperm may be derived from both testis and epididymis. The pI variants are likely to be a result of different carbohydrate components in the glycan structure of Spam1 in the two tissues. It is well known that there is tissue-specificity of glycosyl-transferases and glycosidases (Kobata, 1992).

PNA, which preferentially binds to *O*-linked side chains that are present on sperm plasma membranes (Navaneetham et al, 1996) and apical cells (Calvo et al, 2000) recognized a 67 kd molecular mass in the epididymis, testis, and in caudal sperm. Evidence that this 67 kd membrane protein may be Spam1 was obtained by showing that a potential *O*-linked glycosylation site at Thr379 in mouse Spam1 that we identified from a computer analysis of Spam1 cDNA sequence (Deng et al, 1999) is functional (Figure 3). It is noteworthy that recently, Baker et al (2002) identified a 68 kd PNA-staining sperm membrane protein that localizes to the acrosomal crescent and the principal piece where we have localized Spam1 (Deng et al, 1999).

Our results demonstrate that the lectins PHA-E, LEL, and STL, which bind to *N*-linked side chains and which were also shown to stain PH-20 from macaque sperm (Li et al, 2002), identified a 67 kd protein molecular mass in epididymis and caudal sperm, but not in testis. Whereas LEL and STL have an affinity for *N*-acetyl glucosamine, PHA-E has a specificity for complex structures. Thus the findings in this study indicate that testicular and epididymal Spam1 glycoforms, which contain an identical peptide core, are differentially glycosylated: in the epididymal glycoform *N*-acetyl glucosamine is a major sugar within or at the terminal end of one or more linked glycans. Differential glycosylation has been reported for at least two other epididymal proteins: CD52 (SAGA-1), in which the difference is between lymphocytes and epididymis (Diekman et al, 1997); and clusterin (Ahuja et al, 1996), in which the difference is between testicular and epididymal glycoforms.

The 67 kd band for the different *N*-linked lectins was verified to be Spam1 by two methods. First, lectin blot analysis of two-dimensional SDS-PAGE of protein in epididymal tissue and luminal fluid identified isoforms at 67 kd molecular mass with pIs ranging from 7.6 to 8.3. This correlates with the results from Western blot analysis of two-dimensional SDS-PAGE in epididymal tissue and luminal fluid. Second, the LEL bands at 67 kd in epididymis and sperm were confirmed to be Spam1-specific with the use of anti-Spam1 serum. After preincubating protein extracts from epididymis and caudal sperm with preimmune serum or anti-Spam1 serum, the lectin bands disappeared in the latter indicating Spam1 specificity. It must be pointed out that Spam1 *N*-linked sites (Asn46, 165, 293, and 401) are evenly spaced (Deng et al, 1999) and that the antipeptide Spam1 that was used is generated from a 15-mer at positions 381–395, which would be between Asn293 and Asn401. It is possible that steric hindrance from the antibody binding could be responsible for occluding the *N*-linked chains at Asn293 and Asn401.

N-glycans have been shown to mediate apical sorting of GPI-anchored proteins (Benting et al, 1999), a finding consistent with Spam1 location on sperm and on the apical surface of the epididymis from where it was shown to be released in vivo and in vitro (Deng et al, 2000; Zhang and Martin-DeLeon, 2001). Our finding that three lectins specific for *N*-linked glycans stain a protein identified as Spam1 from the epididymis and caudal sperm, but not testis, suggests the following: 1) TS on sperm may undergo deglycosylation during epididymal transit to generate *N*-linked sites not found on the protein in the testis (Deng et al, 1999), and 2) ES may be directly involved in the *N*-linked sites on sperm by either functioning as the glycosidase (in the luminal fluid) involved in the deglycosylation process or by binding to sperm. It is noteworthy that Baker et al (2002) identified a 68 kd mouse sperm membrane protein that stained with LEA (LEL)

and showed it to be located on intact sperm in a pattern distinctly different from that of PNA. It was seen on the anterior crescent and the posterior head where we have localized Spam1 (Deng et al, 1999). The similarities in molecular weights, staining, and localization of Spam1 and that of the protein described by Baker et al (2002), strongly argue that the latter is Spam1. The staining distribution reported by Baker et al (2002) was seen on some sperm but not on others, suggesting that it may be associated with epididymal maturational states and implicating the involvement of ES.

Epididymal Spam1 Is Released in Epididymosomes (Exosomes) With its Lipid Anchor

After ultracentrifugation of the luminal fluid, Spam1 was found to be present in both the supernatant and the pellet although preferentially in the latter. This indicates that there are two populations of this membrane protein, as is the case for epididymal protein DE in rats (Cohen et al, 2000) and for prostasome-like particles in rat epididymal fluid (Fornes et al, 1995). However, it is possible that there may be two sources of Spam1 in the luminal fluid: molecules that come from sperm and those that are released from the epididymis. Because ES was observed in vesicles that were seen in the process of being released in the lumen (Deng et al, 2000), it is likely to reside in the insoluble pellet. This insoluble membranous material, which is similar to prostasomes, has been referred to as epididymosomes and serves as a means of transferring proteins to the sperm surface (Rooney et al, 1996; Yeung et al, 1997).

We have demonstrated that ~60% of the Spam1 from the insoluble particles obtained after the initial ultracentrifugation of the luminal fluid was released into the medium following 15 minutes of exposure to PI-PLC (Figure 5, E and F). This confirms that ES is secreted into the lumen via lipid vesicles or epididymosomes, probably as a complex of GPI-linked proteins and signaling molecules. Our inability to release the lipid anchor in 100% of Spam1 molecules might result from the inaccessibility of some of the molecules to the enzyme, or it could indicate the presence of a population of Spam1 that is either not PI-anchored or has a GPI anchor that is insensitive to the particular PI-PLC (Cooper, 1998).

GPI-anchored proteins are often known to be resistant to detergent extraction (Triton X-100) and are referred to as detergent-insoluble glycolipids, which float on top of sucrose gradients (Primakoff et al, 1988; Cherr et al, 2001). However, 100% of the Spam1 from the insoluble particles was extracted by exposure to 1% Triton X-100 in our study (Figure 5, G and H), supporting the evidence that ES is released with its lipid anchor, a form in which it can bind to sperm.

Our studies, which provide further evidence that Spam1 is produced independently in the testis and epididymis, reveal that TS and ES are different glycoforms and that the latter shares identical glycans with caudal sperm. There is therefore evidence to suggest that ES may not be a redundant protein. The findings of this study implicate an interaction between sperm and ES, similar to other GPI-anchored proteins such as CD52 (Rooney et al, 1996) and P26h, P34H, and P25b (Frenette and Sullivan, 2001).

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