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Epididymal SPAM1 and its impact on sperm function

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Abstract

The most widely conserved mammalian sperm antigen is sperm adhesion molecule 1, SPAM1/PH-20, which is also the major testicular hyaluronidase. This multifunctional glycosyl phosphatidylinositol (GPI)-linked protein plays several roles in fertilization and is encoded by a gene that resides among hyaluronidase family members in a cluster on human 7q31/mouse 6A2. In the human cluster, *SPAM1* is the only functional hyaluronidase and of all six hyaluronidases in the genome it is the best characterized, both structurally and functionally. While *SPAM1* transcripts are abundantly expressed only in the testis, specifically in spermatids, the RNA and protein are present in the male reproductive tract and accessory organs and in the female tract of mice. Our investigation of the post-testicular expression of SPAM1 shows that the protein is widely expressed in the epididymis. Like testicular SPAM1, epididymal SPAM1 (ES) has hyaluronidase activity and is conserved in at least five species (mouse, rat, bull, macaque, and human) all of which have putative androgen response elements in the gene promoters, consistent with androgen regulation. Testicular lumicrine factors have also been implicated in ES regulation. Based on regional expression, the protein is likely to play a role in both sperm maturation and storage. A minor secretory glycoprotein, ES is present in the epididymal luminal fluid in both a soluble and insoluble form (epididymosomes), with the latter having an intact lipid anchor. Genetic approaches have provided evidence for sperm uptake of ES in vivo, and in vitro uptake has been demonstrated with the use of *Spam1* null mice. In vitro acquisition of ES on the sperm surface results in a pattern that mimics the wild-type distribution. More importantly it significantly increases the ability of null sperm to penetrate the cumulus of oocytes via hyaluronidase activity, directly relating ES uptake with fertilizing ability and indicating that ES is a marker of sperm maturation.
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Keywords: Secretory protein; Epididymosomes; Sperm maturation; Hyaluronidase activity

1. Introduction

Mammalian genomes contain two clusters of tightly linked hyaluronidase (hyase) genes of which the best characterized member, both structurally and functionally, is *sperm adhesion molecule 1* (*SPAM1*). The protein, SPAM1 (PH-20), encoded by this gene which resides in the reproductive cluster on human 7q31/mouse 6A2 (Fig. 1) is the most widely conserved mammalian sperm membrane protein (Lathrop et al., 1990). It is known to play multiple essential roles in fertilization (Cherr et al., 2001). Like *SPAM1/Spam1* the other two genes in the human reproductive cluster, *HYALP1/Hyalp1* and *HYAL4/Hyal4*, are abundantly expressed in the testis. However, the latter is actually a chondroitinase without hyaluronidase activity (Csoka et al., 2001; Stern, 2003). Since human *HYALP1* is a pseudogene, *SPAM1* is the only functional hyase in the "reproductive cluster", underscoring its importance in human fertilization.

In sharp contrast the mouse reproductive cluster has three functional hyases, due to the presence of the rodent-specific *Hyal5* and to the fact that *Hyalp1* is functional. Since both humans and mice have three genes (*HYAL1/Hyal1, HYAL2/Hyal2,* and *HYAL3/Hyal3*) in the somatic or ubiquitous cluster (which is highly expressed in a variety of tissues) on human 3p21/mouse 9F1, the total number of murine hyase genes is seven compared to six in humans (Csoka et al., 2001). In the mouse where *Hyal5* shares 71% and 60% homology at the nucleotide and amino acid level with *Spam1*, it has been thought to be functionally redundant to *Spam1*, compensating for it in *Spam1 null* mice which are fertile with only subtle phenotypic effects (Baba et al., 2002). However, our recent structural and functional characterization of both Hyal5 and Hyalp1 suggests that either or both could functionally compensate for Spam1 in *Spam1* null mice (Zhang et al., 2005; Miller and Martin-DeLeon, in preparation). Thus, the three murine reproductive hyases which show strong structural similarities have overlapping and redundant functions which are likely to be performed solely by human SPAM1. Therefore the functional redundancy in the mouse reaffirms the biomedical significance of the mouse model for gaining an understanding of human SPAM1.

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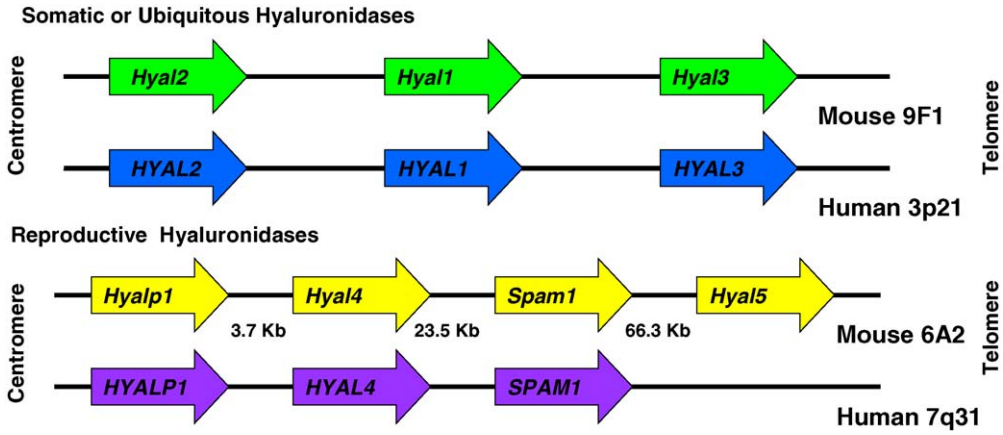

Fig. 1. The mammalian hyaluronidase gene clusters in mice and humans.

1.1. Structure and functions of SPAM1

A sperm antigen, SPAM1 is found on both the plasma membrane and the inner acrosomal membrane (Myles and Primakoff, 1997). The molecular structure of SPAM1 has been elucidated: it is a single chain glycoprotein with a glycosyl phosphatidylinositol (GPI) anchor (Fig. 2). Although the MW of the protein is 80 kDa in bulls (Morin et al., 2005), it is −64 to 68 kDa in a variety of other species such as humans (Sabeur et al., 1997), guinea pigs (Phelps and Myles, 1987), macaques (Vines et al., 2001) and mice (Thaler and Cardullo, 1995; Deng et al., 1999). Variations in MWs may be attributed to endoproteolytic cleavage of the molecule as well as different glycosylation levels, since the protein is glycosylated with both *N*- and *O*-linked sites (Lathrop et al., 1990; Deng et al., 1999).

The functional domains of SPAM1 in mice and primates can be seen in Fig. 2. A multifunctional protein, SPAM1 has been shown to play a role in at least three important functions during fertilization: (1) cumulus dispersion due to the insoluble hyase activity at neutral pH [domain NH; Lin et al., 1994; Hunnicutt et al., 1996]; (2) secondary zona pellucida binding after the acrosome reaction [domain ZP; Cherr et al., 2001; Myles and Primakoff, 1997], and 3) Ca^{2+} signaling-associated acrosomal exocytosis mediated by the HA-binding receptor [domain HA; Vandevoort et al., 1997; Sabeur et al., 1998; Cherr et al., 1999]. The acidic hyase activity [domain AH] is present in soluble SPAM1 that is generated after the acrosome reaction (AR), after cleavage at the carboxy terminus (Hunnicutt et al., 1996) and may be involved in the penetration of the zona and the perivitelline space by acrosome–reacted sperm (Talbot, 1984; Dandekar and Talbot, 1992; Zaneveld and De Jonge, 1991).

1.2. Dual expression of SPAM1 in testis and epididymis

While *SPAM1* is abundantly expressed only in post-meiotic germ cells of the testis (Phelps and Myles, 1987; Jones et al., 1996; Zheng et al., 2001a; Fleming et al., 2004; Morin et al., 2005) where the RNA is detected with Northern analysis (Jones et al., 1995; Zheng and Martin-DeLeon, 1997), its expression also occurs in diploid cells where the transcript is less abundant. Using the more sensitive techniques of RNase protection assay (Martin-DeLeon et al., 2005a; Hyaluronan 2003, The Cleveland Clinic and Matrix Biology Institute, #35 Oct 11-16, Cleveland, OH), in situ transcript hybridization and RT-PCR (Deng et al., 2000; Evans et al., 2003; Zhang et al., 2004) the transcripts have been found throughout the epididymis, where the protein is also present.

The protein was first shown to be present in membranous vesicles in the principal cells of the epithelium in the caput, corpus and cauda in sperm-free epididymal tissues, from germ

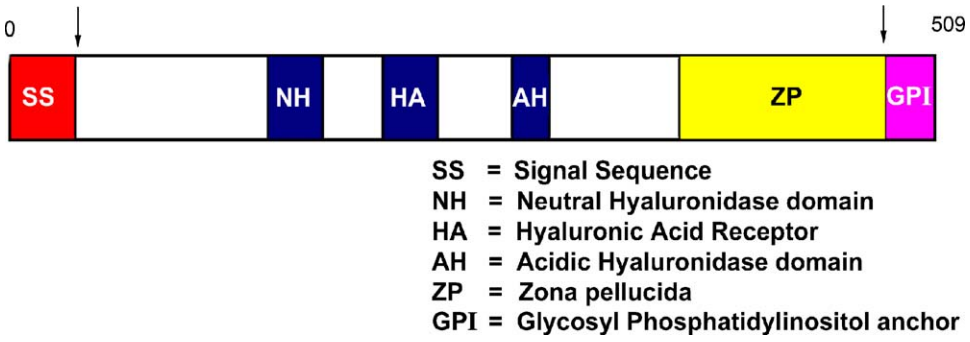

Fig. 2. SPAM1 is a single chain glycoprotein that is GPI-linked. Arrows near the N- and C-termini indicate the cleavage sites for the signal sequence (SS) and the GPI anchor. Catalytic sites for neutral and acidic hyase activity are NH and AH, respectively; and they flank the HA-binding site (HA). ZP is the zona pellucida binding domain.

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Table 1
Epididymal SPAM1 is conserved

	Location and relative amounts of expression				
	Caput	Corpus	Cauda	LF	Reference
Mouse	++	+++	++	X	Deng et al. (2000), Zhang and Martin-DeLeon (2001)
Rat	+++	++	+	N/S	Zhang et al. (2004)
Macaque	+	+++	++	N/S	Evans et al. (2003)
Human	+	+++	++	N/S	Evans et al. (2003)
Bull	+	+++	+	X	Morin et al. (2005)

N/S: not studied, LF: luminal fluid, X: present.

cell-deficient mutant and wild-type mice, expressing *Spam1* RNA (Deng et al., 2000). Confirmation of the independent expression of Spam1 in the murine epididymis and testis came from studies of co-culture of corpus and cauda epithelial cells with their corresponding epididymal fibroblasts (Zhang and Martin-DeLeon, 2001). That study also showed that epididymal Spam1 was released in the medium of cultured epithelial cells, as well as in vivo in the luminal fluid from all three epididymal regions. Evidence documenting both the release of the protein and its independent expression in the testis was later provided from studies of efferent duct ligated (EDL) rats (Zhang et al., 2004).

Epididymal expression of *SPAM1* has now been reported in five mammalian species; namely, mouse, rat, bull, macaque and human (Table 1) where both the RNA and protein have been observed throughout the organ. The evolutionary conservation of the dual expression pattern in testis and epididymis underscores its functional importance. In all species, expression in the epididymis is confined to the principal cells of the epithelium which was studied in humans using laser capture microdissection/RT-PCR and in situ transcript hybridization (Evans et al., 2003). The level of expression of SPAM1 is region-dependent and varies among the species: the highest level is seen in the corpus for all species studied to date, except the rat where it occurs in the caput. Here, the lowest level is seen in the cauda (Table 1). The observation of the two patterns is consistent with the findings of Turner et al. (2003) that epididymal function is regulated within segments which vary among species. The expression patterns suggest that epididymal SPAM1 is likely to play a more prominent role in sperm maturation, which occurs in the caput and the corpus (Soler et al., 1994), than in storage in the cauda where sperm viability is maintained (Moore, 1996).

In all species studied, epididymal SPAM1 shares approximately the same molecular weight as testicular SPAM1 and demonstrates neutral hyaluronidase activity in all the three regions of the organ (Deng et al., 2000; Zhang and Martin-DeLeon, 2001, 2003a; Evans et al., 2003; Morin et al., 2005). Only marginal acidic hyaluronidase activity could be demonstrated (Deng et al., 2000). An interesting finding is that in both normal and EDL rat tissues, hyaluronidase activity levels for caput and cauda are inversely related to protein levels; these are lowest in the cauda and highest in the caput (Zhang et al., 2004) (Table 1). The increased hyase activity in the cauda of EDL rat is likely not to be due to differences in the level of glycosylation, which has been shown to influence hyase activity in caput and caudal sperm (Deng et al., 1999; Rutllant and Meyers, 2001), since the MW of the protein is unaltered in the different epididymal regions (Zhang et al., 2004). It is possible that related hyases might be responsible for the increased activity in the cauda, since the rodent-specific Hyal5 has been shown to be highly expressed in the cauda in mouse (our observation).

2. Regulation of expression of *SPAM1*

2.1. Transcriptional and posttranscriptional control of SPAM1 expression

Most of the studies on the regulation of expression of SPAM1 have been performed in the mouse. Studies on the transcriptional regulation of *Spam1* indicate that epididymal and testicular *Spam1* are under different control, as they have different RNA transcription initiation sites (Zhang and Martin-DeLeon, 2001). However, this difference does not alter the size of the protein in the two tissues, since the translational start site is present in exon II (Zheng and Martin-DeLeon, 1999). The promoter region of *SPAM1* in the mouse and human has been cloned and analyzed (Zheng and Martin-DeLeon, 1999; Evans et al., 2003), and the presence of various putative transcription factor-binding sites is consistent with the expression of the gene in testis and epididymis. Spermatid expression is driven by a cyclic AMP responsive element (CRE), found in both species and shown to be functionally active in the mouse (Zheng and Martin-DeLeon, 1999). It is generally known that gene expression in the epididymis is driven by androgen responsive elements (AREs) to which androgen receptors, stimulated by androgens, bind (Robaire and Viger, 1995; Schwidetzky et al., 1997). Not only have putative AREs been identified in the species expressing ES, but their relative location with respect to CRE is common. The AREs are distal to CRE and this pattern seems to be consistent for genes that are dually expressed in testis and epididymis (Hsia et al., 2002).

There is evidence for posttranscriptional regulation of *SPAM1* RNA. While testicular and epididymal transcripts have the same polyadenylation sites (in that there are no alternative polyadenylation sites in the RNA sequence), poly(A) tails of epididymal *Spam1* RNA are significantly shorter (Zhang and Martin-DeLeon, 2003a). Shortened poly(A) tails are decay intermediates associated with distributive deadenylation. This is the first step in mRNA decay which is mediated by *cis*-acting AU-rich elements (AUREs) in the 3′UTR (Tourriere et al., 2002; Hamilton et al., 1993). These elements are targeted by *trans*-acting cytoplasmic proteins to mediate (in)stability of the RNA (Bevilacqua et al., 2003). Our recent finding that the 3′UTR of *Spam1* contains *cis*-acting AU-motifs that are targeted by *trans*-acting proteins (Martin-DeLeon et al., 2005b) which are tissue-specific (our observation) suggests differential posttranscriptional regulation of *Spam1* RNA stability in testis and epididymis.

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2.2. Possible regulation by testicular lumicrine factors

There is evidence that testicular lumicrine factors may regulate the expression of ES. In W^{bb} sperm-free mutant mice with pre-meiotic arrest, ES is drastically reduced, particularly in the caput and cauda, when compared to sperm-free Rb(6.16)/Rb(6.15) double heterozygotes where the spermatogenic arrest occurs during the meiotic and post-meiotic stages (Deng et al., 2000). This observation suggests that germ cell-associated factors may be responsible for regulating the expression of ES in caput and cauda. This conclusion of an involvement of testicular lumicrine factors in the regulation of ES is strongly supported by studies of EDL rats.

In EDL rats where the epididymis is androgen-maintained and where sperm, Sertoli cell-derived factors, and germ cell-associated factors are prevented from interacting with the epididymal epithelium, the processing of ES is abnormal. In non-EDL epididymis that is made sperm-free by washing the tissues, ES has the same isoforms as found on sperm in the corresponding epididymal regions. However, this is not the case for ES taken from EDL rats (Zhang et al., 2004). In the latter, where large amounts of a >100 kDa protein were found in the caput and corpus tissues under non-reducing conditions, there were a −50 kDa and a 28 kDa band after reduction, neither of which is seen in normal tissues or on sperm (Zhang et al., 2004). Additionally, there was a marked increase in the amount of ES in EDL compared to non-EDL rats. This suggests that not only the processing, but also the amount of protein may be regulated by interactions of testicular lumicrine factors with the epididymal epithelium. These factors may be comprised of inhibitory components in the rete testis fluid, or it is possible that a feedback control may be involved in expression. Either of these suggests the existence of cross-talk between epididymal tubule epithelium and testicular lumicrine factors. Further studies are necessary to determine if the increased amount of ES in EDL rats is due to an increased rate of mRNA synthesis or the accumulation of the protein in the absence of uptake by sperm.

2.3. Epididymal SPAM1 is a secretory protein

In considering the role(s) of ES, it is important to consider the nature of the release of the protein from the principal cells, which likely occurs via blebbing or apocrine secretion (Hermo and Jacks, 2002). In the mouse and bull, release has been documented by the presence of the protein in the luminal fluid (Zhang and Martin-DeLeon, 2001, 2003a; Morin et al., 2005) and in conditioned media of cultured epididymal epithelial cells (Zhang and Martin-DeLeon, 2001). In EDL rats, where luminal fluid was not studied, there was a build-up of SPAM1 in the lumen in the absence of sperm (Zhang et al., 2004). As is the case for a number of epididymal proteins (Cohen et al., 2000; Fornes et al., 1995), Spam1 in the epididymal luminal fluid has been found as partly insoluble vesicular epididymosomes and partly in a soluble form, after fractionation by ultracentrifugation (Zhang and Martin-DeLeon, 2003a). Epididymosomes are extracellular structures, which commonly transfer proteins to spermatozoa (Yeung et al., 1997; Saez et al., 2003). They are thought to originate from apical blebs which protrude from the principal cells in the lumen and are eventually released in the form of apocrine secretion (Hermo and Jacks, 2002).

We have shown that ES is secreted with its lipid anchor intact, a form in which it can bind to sperm, and that it lies on the outer surface of the epididymosomes where it can be detached by enzymatic cleavage (Zhang and Martin-DeLeon, 2003a). Thus, the possibility exists that ES-associated epididymosomes may be involved in posttesticular maturation of sperm during epididymal transit and storage, similar to other GPI-anchored proteins such as CD52 (Rooney et al., 1996) and P26h, P34H, and P25b (Frenette and Sullivan, 2001).

2.4. Indirect evidence for sperm acquisition of ES in vivo

During storage in the cauda, sperm are exposed to a microenvironment that is rich in epididymal secretions and that impacts their maturational status. Mice bearing the Rb(6.16) or Rb(6.15) Robertsonian translocation (Rb), each of which carries a different mutant *Spam1* allele (Zheng et al., 2001b), have provided indirect evidence that Spam1 quantities are increased during sperm epididymal transit and storage. Due to the dysfunction of the Rb-bearing sperm in carriers, instead of the Mendelian 1:1 segregation ratio in the progeny, there are transmission ratio distortions (TRDs) in favor of the wild-type allele. Using a regime with different mating intervals, the TRDs for Rb(6.16)/+ and Rb(6.15)/+ were 3.6:1 and 2.4:1 when sperm were stored physiologically for 3 days, and 2.1:1 and 1.8:1 after a 14-day storage (Aranha and Martin-DeLeon, 1991, 1995; Chayko and Martin-DeLeon, 1992). These changes reflect an improvement in the fertilizing ability of the Rb-bearing sperm with prolonged storage (Table 2). The striking reductions in TRDs seen after physiological storage are bolstered by findings for sperm experimentally isolated in the cauda. In Rb(6.16)/+ the TRD was eliminated when sperm were surgically isolated in the cauda for 6–8 days prior to mating (Aranha and Martin-DeLeon, 1992), as seen in Table 2. The elimination of the TRD suggests an enhancement of the quantities of Spam1 and related hyases on the Rb-bearing sperm with storage time. [We have recently shown that the Rbs also carry mutations of the closely linked and closely related *Hyal5* and *Hyalp1* which have similar expression patterns and overlapping functions with *Spam1* (Zhang et al., 2005), and which could contribute to the sperm dysfunction leading to reduced fertility and TRD seen in these mice (Zheng and Martin-DeLeon, 1997; Zheng et al., 2001a).]

In mice carrying a *Spam1* mutation (Baba et al., 2002) the results of flow cytometric analysis of caput and cauda sperm are indicative of Spam1 acquisition during epididymal transit. Due to a lack of transcript sharing in spermatids (Zheng et al., 2001a; Martin-DeLeon et al., 2005b), there is a bimodal distribution of Spam1 in caput sperm from *Spam1* +/− males. However, normal distributions with higher intensities, reflecting increased Spam1 quantities, are seen for caudal sperm in these mice (our observations).

Other lines of evidence suggesting that ES can be acquired by sperm, come from examining the biochemical maturation

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Table 2
Indirect evidence for sperm acquisition of ES

Species	Observation	Reference
Mouse	Rb/*Spam1* mutants—Change in TRD[a] 3-day storage 3.6:1 and 2.4:1 14-day storage 2.1:1 and 1.8:1	Aranha and Martin-DeLeon (1991, 1992, 1995), Chayko and Martin-DeLeon (1992)
	Surgical isolation of cauda 1:1	
	Spam1 +/− Change in distribution on caput to caudal sperm	Griffiths and Martin-DeLeon (in preparation)
	Wild-type—lectins in sperm and ES are identical, −4 × increase in caudal compared to caput sperm	Zhang and Martin-DeLeon (2001), Deng et al. (1999)
Stallion	Increase in cauda vs. caput sperm	Rutllant and Meyers (2001)
Bull	Increase in cauda vs. corp. vs. caput sperm	Morin et al. (2005)
Rat	Accumulation of ES in lumen in absence of sperm in EDL males	Zhang et al. (2004)

[a] TRD: transmission ratio distortion.

of the protein during sperm epididymal transit in wild-type animals. In mice, caudal sperm were shown to have a >four-fold increase of Spam1 and hyaluronidase activity, compared to caput sperm (Deng et al., 1999). Similarly an increase of SPAM1 in caudal sperm, compared to caput ones, has been observed in stallions (Rutllant and Meyers, 2001) and more recently in bulls (Morin et al., 2005). In bulls where sperm were examined in all three regions of the epididymis there was a progressive increase of p80 (SPAM1) from caput to cauda, although the increased accessibility of the antigen as the basis for the increased intensity of the signal could not be ruled out (Morin et al., 2005). Additionally, mouse sperm Spam1 and ES share identical *N*-linked carbohydrate moieties which are not present in testicular Spam1 (Zhang and Martin-DeLeon, 2003a) (Table 2). This observation is bolstered by the finding that 2D PAGE and immunoblots revealed overlapping isoforms for Spam1 from the testis, epididymis and caudal sperm (Zhang and Martin-DeLeon, 2003a). Finally, there is compelling evidence for sperm acquisition of ES from EDL rats. In the absence of sperm in ligated ducts there is a marked accumulation of ES in the lumen, and this is accompanied by a similar increase in hyase activity, both of which are not seen in normal rat tissues (Zhang et al., 2004). While the accumulation may be due partly to the absence of inhibition by lumicrine factors, the absence of sperm uptake is highly likely to be contributory.

Overall, these studies suggest that acquisition of ES and related hyases may be one of the modifications that occur to the sperm membrane during epididymal passage. How this may be involved in the progressive migration of the protein on the plasma membrane as it goes from a more distal to a proximal location on the tail (Morales et al., 2004), and from a more uniform to a regionalized distribution on the head (Phelps and Myles, 1987; Deng et al., 1999; Seaton et al., 2000; Cowan et al., 1991) is unknown. Since the rate of diffusion of SPAM1 is only 1/1000th of that for lipids (Cowan et al., 1991; Phelps et al., 1988), the mechanisms that enable membrane proteins to migrate across putative intra-membranous barriers and to undergo lateral diffusion in the lipid bilayer, are unlikely to be involved in its re-distribution on the plasma membrane. It is therefore possible that binding of ES to the sperm surface may play a role in the re-distribution of the protein.

2.5. Sperm acquisition of ES in vitro and its impact on cumulus penetration

Direct evidence that ES can be acquired by sperm has come from in vitro sperm binding studies using genetic mutants (Griffiths and Martin-DeLeon, in preparation). When sperm from *Spam1* null mice were used for binding assays with sperm-free unfractionated luminal fluid from wild-type mice, immunocytochemistry revealed Spam1 uptake on the sperm surface in a pattern that mimics that for wild-type sperm. Uptake was confirmed by flow cytometric analysis which provided a quantitative assessment of a large population of sperm, and showed heterogeneity in the distribution. This heterogeneity reflects two subpopulations of sperm, those with and without binding. To address the issue of uptake under near saturation conditions, sperm from the homozygous Rb(6.16)-*Spam1* mutant where the amount of Spam1 is −70% of wild-type (Zheng et al., 2001b) were used for binding assays. The results showed a substantial level of binding (Griffiths and Martin-DeLeon, in preparation). These results which provide direct evidence for sperm uptake of epididymal Spam1 are supportive of in vivo binding physiologically, during sperm maturation and storage.

When the impact of binding was determined by the efficiency of sperm to effect cumulus penetration under IVF conditions after uptake, a significant improvement in the penetration rate of *Spam1* null sperm was observed. Null sperm incubated in wild-type epididymal luminal fluid, compared to those incubated with null or wild-type luminal fluid treated with Spam1 antibody, had a significant increase in penetration efficiency after 7 h, $P < 0.001$, Fisher's exact test. Importantly, aliquots of the null sperm used in the penetration assay were demonstrated to bind Spam1 under IVF conditions, directly relating binding to penetrating ability (Griffiths and Martin-DeLeon, in preparation). These results show that sperm binding of ES is associated with an increased ability to traverse the cumulus barrier, one of the first steps in mammalian fertilization. Thus, this sperm surface protein can be considered as a marker of sperm epididymal maturation.

2.6. Immunocontraceptive activity of SPAM1

The location of SPAM1 (PH-20) on the sperm surface and its well-defined essential roles in fertilization have made it a target

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Table 3
SPAM1 and Immunocontraception

Species	Immunization			Fertility	Response	Reference
	M	F	Form	Reduction		
Guinea pigs[a]	+	+	Aff. Purif.	+	(1) Caudal sperm depletion (2) Autoimmune orchitis	Primakoff et al. (1988), Tung et al. (1997)
Rabbit	+	+	rSPAM1	–	Restricted entry of IgG in reproductive tracts	Pomering et al. (2002)
Macaque	+	+	rSPAM1	–	”	Deng et al. (2002)
Mouse	+	+	rSpam1	–	”	Hardy et al. (2004)

M: male, F: female, Aff. Purif.: affinity purified, rSPAM1/Spam1: recombinant SPAM1.
[a] The guinea pig may be fundamentally different from other species in its autoimmune response.

for contraceptive vaccines (Table 3). Immunization with a single inoculation of affinity purified guinea pig SPAM1 resulted in 100% reversible infertility in both males and females (Primakoff et al., 1988; Tung et al., 1997). When the mechanism of infertility induction in males was investigated, it was shown that infertility resulted partly from experimental autoimmune orchitis and partly from a complete loss of normal sperm from the cauda. The latter was due to crude testis antigens other than PH-20 (Tung et al., 1997). Attempts at using SPAM1 as a contraceptive vaccine in other species have produced different results. When bacterially produced recombinant rabbit PH-20 was used for immunization, there was a lack of contraceptive effect in rabbits (Holland et al., 1997). The lack of sterility resulted from insufficient PH-20 antibodies in the male and female reproductive tract due to restricted entry of IgG (Pomering et al., 2002).

In a primate model, *Cynomolgus macaques*, combinations of adjuvant and antigens derived from synthesized and recombinant proteins all produced significant immune responses in females, with circulating antibodies recognizing macaque sperm surface PH-20 (Deng et al., 2002). However, there was a lack of sterility following immunization in this model also (Deng, personal communication). Finally, in a study designed to assess fertility control, using disseminating virally vectored contraceptive vaccines, recombinant mouse PH-20 was studied. Mice immunized with either recombinant murine cytomegalovirus expressing PH-20 or directly with the purified recombinant PH-20 developed circulating antibodies, but failed to produce a significant reduction in fertility in males or females when limitations of the delivery systems were unlikely (Hardy et al., 2004). In this regard, it should be noted that Spam1 has been shown to be expressed in the female tract of mice (Zhang and Martin-DeLeon, 2003b) among other tissues (Deng et al., 2000) and this widespread expression in this species could “affect the ability to break tolerance to PH-20 by active immunization” (Hardy et al., 2004).

Taken together, the data from non-primate and primate models (where there are no known redundant reproductive hyases) suggest that PH-20 is not a useful antigen for inclusion in immunocontraceptive vaccines. The absence of critical epitopes in recombinant PH-20 has been considered as an explanation for a lack of sterility after immunization in mice (Hardy et al., 2004). However, it is possible that the strong immunocontraceptive effect reported for affinity purified guinea pig PH-20 could reflect a fundamental difference in the autoimmune response in guinea pigs compared to other species.

2.7. Future work

Currently, very little is known of the mechanism(s) by which Spam1 or other GPI-linked epididymal proteins are transferred to the sperm plasma membrane. While epididymosomes, insoluble membranous vesicles, have been reported to be the common vehicle for transfer (Saez et al., 2003), approximately 40% of Spam1 in the epididymal luminal fluid is soluble and is of the same molecular weight as the insoluble Spam1 which has an intact GPI anchor (Zhang and Martin-DeLeon, 2003a). Does soluble ES also have a GPI anchor which may mediate its transfer to sperm? If so, is there a difference in the efficiency of transfer between the two populations of Spam1? Could the soluble proteins exist in micelles or complexes which could mediate transfer to the sperm membrane? Although hypotheses have been advanced to explain the mechanism(s) of transfer of exogenous GPI-linked molecules to cell surfaces (Ilangumaran et al., 1996), to date there has been no supportive experimental evidence. Understanding the mechanism by which ES is transferred to sperm plasma membrane will fill an important gap in our knowledge, not only of the transfer of reproductive molecules, but of other GPI-linked proteins involved in different physiological processes.

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