

Evolution and function of mammalian binder of sperm proteins

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Abstract Binder of sperm (BSP) proteins are ubiquitous among mammals and have been extensively investigated over the last three decades. They were first characterized in bull seminal plasma and have now been identified in more than 15 different mammalian species where they represent a superfamily. In addition to sharing a common structure, BSP proteins share many characteristics. They are expressed by seminal vesicles and epididymides, interact with similar ligands and bind to the outer leaflet of sperm membranes via an interaction with choline phospholipids. In addition to playing a major role in sperm capacitation, they are implicated as molecular chaperones in sperm motility and viability, in the formation of the oviductal sperm reservoir, in the regulation of cell volume and possibly in the interaction between sperm and oocytes, making them crucial multifunctional proteins. Furthermore, BSP proteins can bind to egg yolk low-density lipoproteins and milk components, an interaction important for the protection of sperm during semen preservation in liquid or frozen state. Our current knowledge of BSP proteins strongly emphasizes their fundamental importance in male

fertility and in the optimization of semen preservation techniques. Much work is still ahead in order to fully understand all the mysteries of BSP proteins.

Keywords Binder of sperm proteins · Sperm · Male genital tract · Functions · Evolution

Introduction

Reproduction is the cornerstone of life and a necessity for the survival of species. It is common knowledge that, in mammals, sexual reproduction involves the interaction and fusion of a male gamete (a spermatozoon) with a female gamete (an oocyte). As simple as it seems, sexual reproduction is in fact a complex series of well-orchestrated maturation steps for both gametes, involving hundreds of external factors. Following their synthesis in the testes, their maturation in the epididymides and ejaculation, sperm are found in a fluid called seminal plasma. This fluid, which is an intricate mixture of secretions from testes, epididymides, seminal vesicles, ampullae, prostate and bulbourethral glands, fulfills critical functions in the maturation, survival and transport of sperm (Rodriguez-Martinez et al. 2011). In addition to lipids, carbohydrates and some minerals, in most species seminal plasma contains more than 1000 proteins playing roles in the regulation of sperm capacitation, the formation of the sperm reservoir, the modulation of uterine immune response, in sperm motility and in the interaction/fusion between gametes (Rodriguez-Martinez et al. 2011).

Amongst the proteins found in seminal plasma, one family of proteins highly conserved in mammals, the binder of sperm (BSP) proteins, has been extensively studied over the past three decades (Manjunath et al. 2009). BSP proteins were first identified in the bull seminal plasma due to their stimulatory

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and inhibitory actions on the release of pituitary gonadotropins (Manjunath 1984). They have since been identified in more than 15 species and have been implicated in a plethora of functions (Plante and Manjunath 2014; Plante and Manjunath 2015a). The current paper aims to be an extensive review of the knowledge gained on BSP proteins since they were first discovered, focusing mainly on their structure, expression and evolution, their binding properties, their connection to semen preservation and their involvement in biological functions.

Structure, expression and evolution

BSP structure

BSP proteins are typically known for their interaction with sperm membrane via choline phospholipids (PC) and their role in sperm capacitation. These proteins are mostly acidic and relatively small with molecular weights ranging from 15 to 30 kDa. Depending on the species, between one and six forms of the protein are expressed. Currently, genes and proteins of the BSP superfamily have been identified in multiple mammals including bulls, boars, stallions, rams, goats, bison, buffalos, humans, chimpanzees, rats, mice, rabbits, alpacas and camels (Bergeron et al. 2005; Boisvert et al. 2004; Calvete et al. 1995b; Druart et al. 2013; Fan et al. 2006; Harshan et al. 2009; Manjunath and Sairam 1987; Manjunath et al. 1987; Sanz et al. 1993; Villemure et al. 2003). They all have in common a conserved structure composed of an N-terminal domain variable in composition and in length, followed by two fibronectin type II domains arranged in tandem (Fn2-A and Fn2-B) separated by a 7-amino acid linker (Fig. 1a). Some of the BSP proteins also possess a short, variable C-terminal domain (Manjunath et al. 2009).

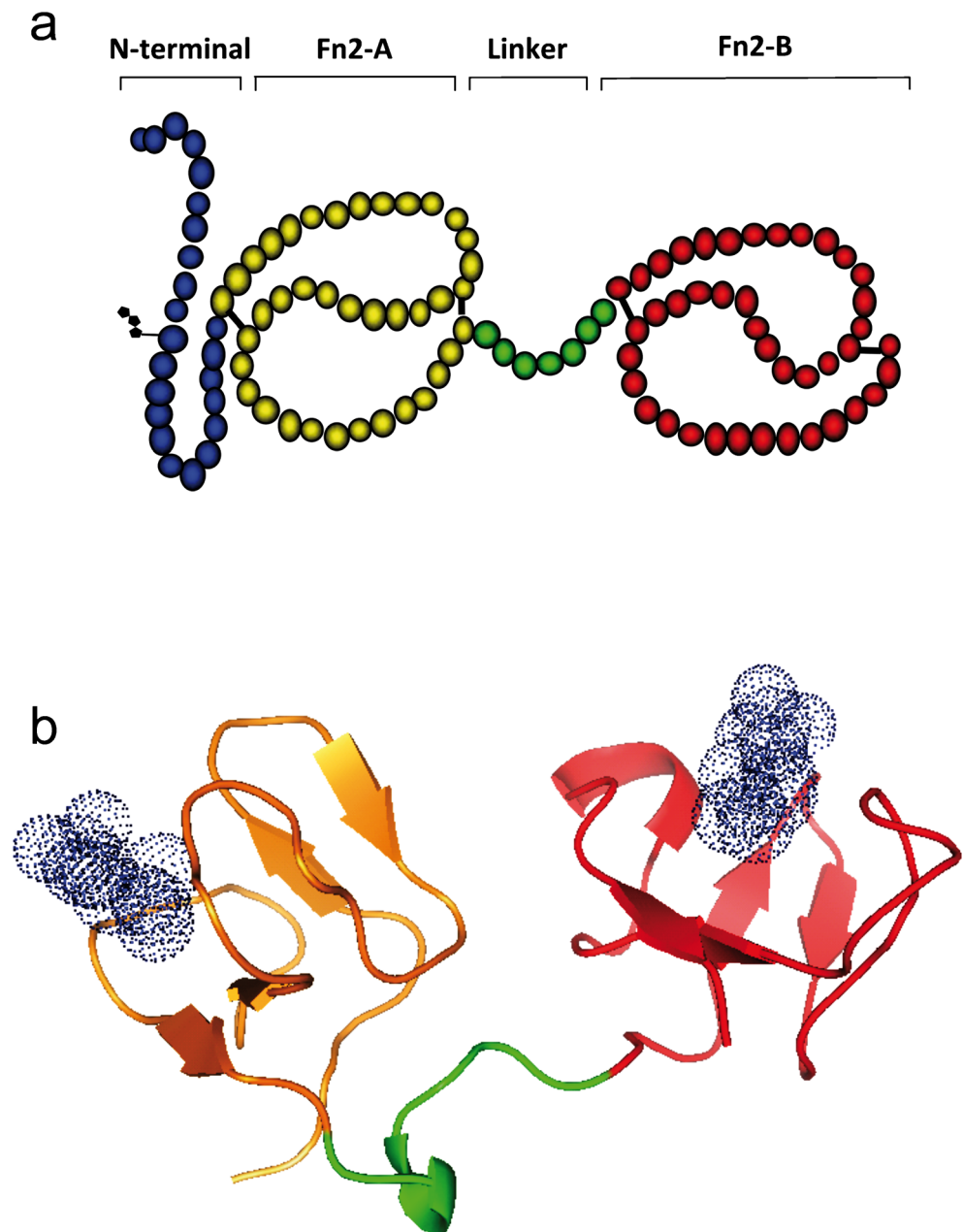
The two fibronectin domains act as functional units for the BSP proteins. Their primary structure contains eight cysteine residues, which are responsible for the formation of four disulfide bonds (two in each domain) with connectivities 1–3 and 2–4, a structure characteristic of the type II domains of fibronectin (Fn2) (Skorstengaard et al. 1984). These bonds result in proteins that adopt a conformation under which each Fn2 domain is composed of two anti-parallel β -sheets connected by an α -helix (Fig. 1b) (Baker 1985; Constantine et al. 1991, 1992; Seidah et al. 1987; Wah et al. 2002). This structure forms a hydrophobic pocket inbetween the two domains. Even though many proteins in mammals possess Fn2 domains, the fibronectin domains found in BSP-related proteins are quite distinctive from the others (Fan et al. 2006). Analysis of the sequences of members of the BSP superfamily showed that these proteins share many conserved motifs especially surrounding the eight cysteines. One of the signature sequences unique to the BSP-Fn2 domains is the presence of a

C-X-F-P-F motif found at the first cysteine residue of each Fn2 domain, where X is either a valine or a non-polar amino acid (Lefebvre et al. 2007).

As opposed to what is observed in the Fn2 domains, the two other fragments of BSP proteins are less conserved between the different homologs. The C-terminus tail is present only in some of the BSP proteins and is usually very short (between 1 and 5 residues). On the other hand, the N-terminal domain varies from 15 up to 71 amino acid residues. BSP1 from rabbit differs immensely from the rest of the BSP superfamily as its N-terminal domain is composed of 380 amino acid residues. No specific function has been attributed to C- and N-terminal parts of BSP proteins (Jois et al. 2015). On the contrary, the N-terminal domain of the BSP proteins has been shown to be intrinsically disordered (Jois and Manjunath 2010). This claim is further supported by the fact that the proposed disordered regions of the bovine BSP1 protein correspond to the missing electron density region in its crystal structure (Fig. 1b) (Wah et al. 2002).

Many BSP proteins are o-glycosylated on threonine residues found in their N-terminal segment (Barrios et al. 2005; Calvete et al. 1995a; Calvete et al. 1997; Manjunath et al. 1988; Serrano et al. 2013). The level of glycosylation and the number of glycosylated residues vary from one protein to another. The most glycosylated protein identified is the bovine BSP5 protein, which is glycosylated on six residues. The carbohydrate chains of BSP proteins contain neutral sugars, galactosamine and sialic acid in varying ratios and linkages (Calvete et al. 1995a; Manjunath et al. 1988). The only oligosaccharide structures resolved to this day are the sugar chains of stallion and bovine BSP1 found on Thr22 and Thr11, respectively. Both proteins have been found to contain one single NeuNAc(α 2-3)-Gal(β 1-3)-GalNAc chain (Calvete et al. 1994, 1995a; Gerwig et al. 1996). The glycosylation of bovine BSP1 is so significant that it can visually be distinguished as two protein isoforms by immunoblot analysis. These proteins, previously designated as BSP-A1 and BSP-A2, are encoded by one gene *Bsp1* in bovine (Manjunath et al. 2009). The exact role of the BSP glycosylation is currently unknown, but in stallion, it was suggested that it could be responsible for the modulation of BSP protein aggregation (Calvete et al. 1995a). Indeed, BSP proteins in bulls, stallions, boars and humans tend to form aggregates in solution (Calvete et al. 1995b; Gasset et al. 1997; Kumar et al. 2008; Manjunath et al. 1988). These aggregates usually contain only BSP proteins except in boar, where BSP1 is known to form a stoichiometric complex with spermadhesin AQN-1 (Calvete et al. 1997). However, human BSPH1 and bovine BSP3 are not glycosylated (Lefebvre et al. 2007; Manjunath et al. 1988). Therefore, glycosylation cannot be solely responsible for the tendency of BSP proteins to aggregate. Their aggregation state can be modulated by the solution composition

Fig. 1 Representations of the bovine BSP1 protein. **a** Schematic representation of the structure of bovine BSP1 protein (adapted from Manjunath et al. 1988). **b** 3D structure of bovine BSP1 produced based on crystallization data from Wah et al. 2002. Each Fn2 domain is composed of two anti-parallel β -sheets connected by an α -helix. The N-terminal domain is not present in the crystal structure



and pH of their environment, which suggests hydrophobic and ionic interactions (Gasset et al. 1997; Manjunath et al. 1988). It is believed that, at physiological ionic strength and temperature, BSP proteins exist in aggregated forms (dimers to octomers) (Calvete et al. 1995b; Manjunath et al. 1987), but dissociate upon binding to sperm membrane (Gasset et al. 1997; Kim et al. 2010; Wah et al. 2002). Based on experimental observations, BSP proteins have a tendency to accumulate on sperm over time, suggesting that, once BSP proteins are bound to sperm membrane, they can still interact and bind to free BSP proteins through hydrophobic interactions (Bergeron and Manjunath 2004, 2006).

Expression and regulation

It was long thought that BSP proteins were exclusively expressed by seminal vesicles and ampullae of mammals (Manjunath and Sairam 1987; Scheit et al. 1988). Early studies of bovine and equine BSP proteins showed that, indeed, transcripts of bovine were found in seminal vesicles and ampullae, without any expression in tissues such as artery, small intestine, adipose tissues, brain, kidney, heart, lung, large intestine and liver, or in reproductive tissues such as epididymis, testis or prostate (Ekhlasi-Hundrieser et al. 2005; Salois et al. 1999). Then, other BSP proteins were identified in the seminal vesicles of rams, goats, bison and buffalos (Bergeron et al.

2005; Boisvert et al. 2004; Harshan et al. 2009; Villemure et al. 2003). However, a few years later, BSP-related genes and proteins were discovered to be expressed in the epididymis of some mammals (reviewed in Plante and Manjunath 2015a). The first study reporting possible epididymal BSP proteins was published in 2006 and revealed the existence of three new BSP-related bovine genes, two of which were expressed in the epididymis (Fan et al. 2006). Following this first publication, epididymal BSP genes and proteins were found in boars (cauda), rams (cauda), rabbits (corpus), mice (caput) and human (initial segment and caput) epididymides (Ekhlas-Hundrieser et al. 2007; Lefebvre et al. 2007; Nixon et al. 2008; Souza et al. 2012). Interestingly, experiments seemed to show that proteins are expressed in the epididymis at a much lower magnitude than those expressed by seminal vesicles.

Only one study has ever suggested the possibility of BSP proteins being expressed in the female reproductive tract. In 2011, Song et al. reported, based on RT-PCR experiments, that porcine *BSP1* is strongly expressed in the seminal vesicles, prostate, caput and corpus epididymides, testis and ovaries of Meishan pigs, and weakly expressed in cauda epididymides and cervix (Song et al. 2011). However, these results are in contradiction with those reported by Ekhlas-Hundrieser et al. where, by northern blot, porcine BSP1 was found expressed in mid-cauda epididymis and was absent in any other regions of the epididymis (Ekhlas-Hundrieser et al. 2007). Thus, the exact expression sites of *BSP1* in boars should be clarified before concluding that BSP proteins are expressed outside the male genital tract.

Although hundreds of studies have been realized to understand the functions and properties of BSP proteins, considerably less work has been done to understand the regulation of their expression. The few data collected show that BSP proteins are not expressed in 14-day-old calves or in young boars, and that their expression level increases with age following sexual maturity (Scheit et al. 1988; Song et al. 2011). Furthermore, data show that castration of adult boars results in loss of BSP1 expression, which can be restored by androgen supplementation (Ekhlas-Hundrieser et al. 2007). These observations suggest that BSP proteins' expression is most likely regulated by the testes and/or androgens. This hypothesis is also supported by the fact that two recent independent ChIP-seq analyses in mice found that androgen receptor binds one DNA region only 500 bp upstream of the *Bsph2* gene, as well as another region located in between *Bsph1* and *Bsph2* genes in cauda epididymides (Hu et al. 2010; Sahu et al. 2014). Interestingly, the androgen receptors do not bind these two regions when the experiments are performed with prostate samples, where *Bsph1* and *Bsph2* are not expressed (Sahu et al. 2014). The hypothesis of an androgen-regulated expression is, however, contradicted by results in rabbit where castration results in the loss of the expression of BSP1, which

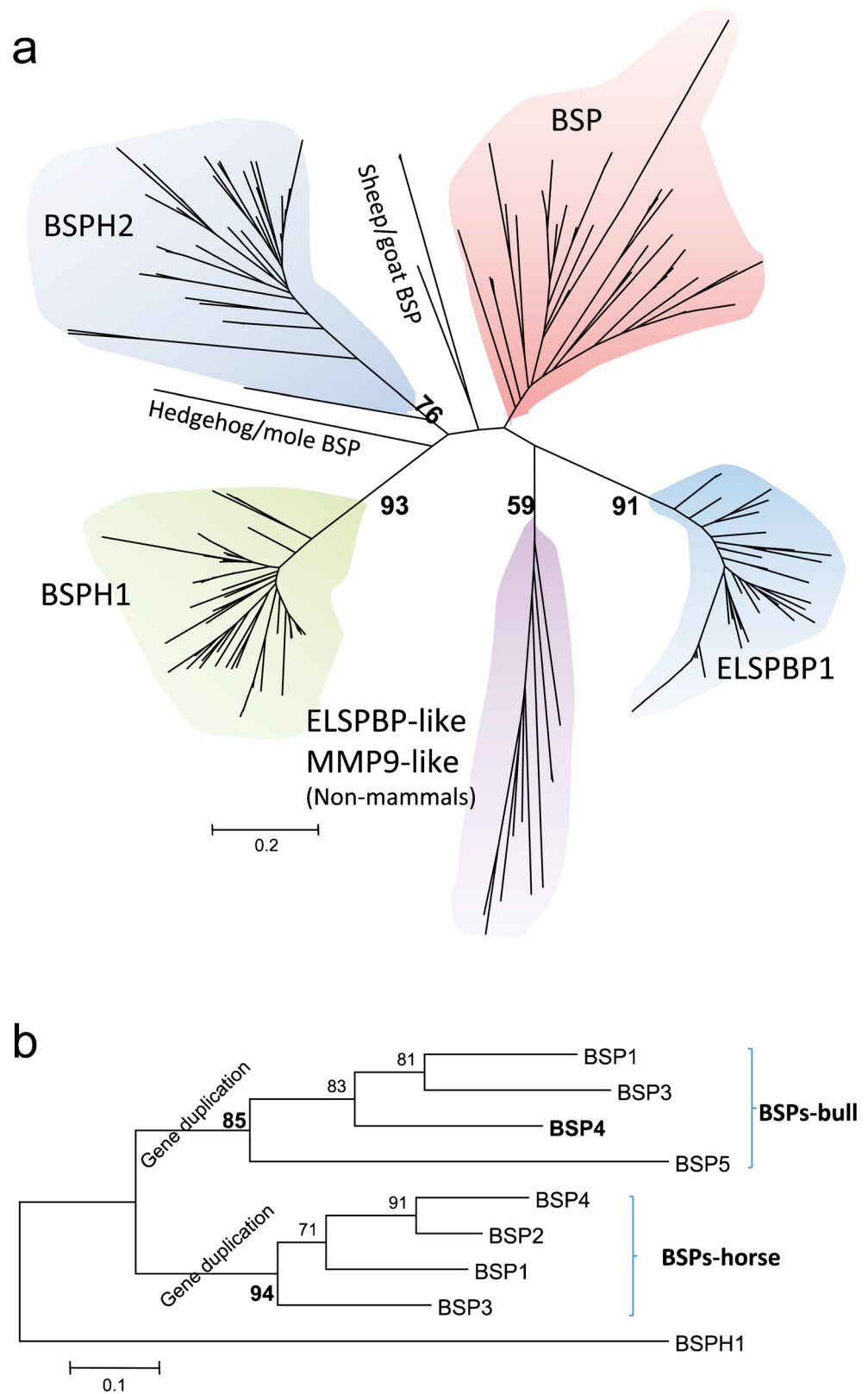
cannot be restored fully by testosterone treatments alone (Nixon et al. 2008). Thus, it appears that BSPs' expression is most likely regulated by testicular factors such as testosterone, but that other factors might also be implicated.

Evolution

As mentioned previously, many proteins containing Fn2 domains can be found in mammals. Phylogenetic studies have shown that Fn2-containing proteins such as BSP proteins, epididymal sperm binding protein 1 (ELSPBP1) or matrix metalloproteinase 9 (MMP-9) most likely evolved by module duplication from a common ancestor (Fig. 2a) (Ekhlas-Hundrieser et al. 2007; Fan et al. 2006; Seidah et al. 1987). However, these families of proteins are all distinct from each other.

These studies also reveal that the BSP superfamily is divided into three subfamilies (BSP, BSPH1 and BSPH2), each containing between one and four bovine *BSP* genes. This finding strongly suggests that gene duplication and diversification have been recurrent in the evolution of BSP genes (Fig. 2a) (Fan et al. 2006). Phylogenetic analyses suggest that the duplication events that gave rise to the three subfamilies occurred before the divergence of the lineages leading to the modern mammals and that the BSP subfamily was probably the most recent to arise since it is absent from primates and rodents (Fan et al. 2006). Therefore, these results suggest that an early duplication of a BSP ancestor gene gave rise to the mammalian BSPH1/BSPH2 subfamilies prior to the divergence of mammals. Later in the evolution of mammals, independent duplication events in ungulates resulted in the emergence of the third subfamily BSP (Tian et al. 2009). Duplication events of BSP genes in individual species occurred subsequently and generated all the different forms of BSP proteins found today (Fig. 2b). Many major differences exist between the BSP subfamily and the two others. For example, genes of the BSP subfamily evolved in time faster than the other two and are under strong positive selection. Genes of the BSPH1 and BSPH2 subfamilies have been found exclusively expressed in the epididymides, whereas genes of the BSP subfamily are more strongly expressed in seminal vesicles. Genes of the BSP subfamily and of the BSPH1 and BSPH2 subfamilies are found in two different conserved regions of the genome. The members of BSPH1 and BSPH2 subfamilies are located between the calcium binding protein 5 (*CABP5*) and the sulfotransferase family 2A (*SULT2A1*) cluster and adjacent with the *ELSPBP1*, which has four FN2 domains. The BSP family members are located between testis expressed 10 (*TEX10*) and LY6/PLAUR domain-containing 3 (*LYPD3*) genes (Tian et al. 2009).

Fig. 2 Phylogenetic relationships of BSP proteins. **a** Phylogenetic relationships of BSP, BSPH1 and BSPH2 families of proteins. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Dayhoff matrix-based method (Dayhoff et al. 1979) and are in the units of the number of amino acid substitutions per site. The analysis involved 238 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 46 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). The bootstrap value over 50 % based on 1000 replicates is shown at each node. Each protein family, BSP, BSPH1, BSPH2 and ELSPBP1 from mammals and some proteins from non-mammals are indicated, where the ELSPBP1-like sequences are distantly related to the FN2-domains from the BSP superfamily. The sequences used in this study were retrieved from the GenBank via BLASTP of non-redundant protein sequences (www.ncbi.nlm.nih.gov) and the top 500 hit sequences were selectively used for the analysis. **b** Neighbor-joining tree of the bovine and stallion BSP genes. The bootstrap values are indicated at each node. Gene duplicate events are indicated. BSPH1 (NP_777267) from bovine was used as out-group. The likely gene duplication events after the divergence of these two species are indicated



It is a sort of diagnostic feature in comparative genomics to determine the origin of the BSP genes. Apparently, many BSP subfamily members are tandemly distributed within the loci

between *TEX10* and *LYPD3* genes, indicating their evolutionary origin by gene duplication, as shown in Fig. 2b. The predicted amino acid sequences of some BSP genes are

apparently in an ambiguous position in the phylogenetic analysis. The dog CDK105L loci, for example, presumably encodes two BSPH1 and BSPH2 genes but is actually clustered into the BSP family, which may suggest that a functional convergency hypothesis would apply here (Fan et al. 2006). In contrast, some BSP subfamily members from sheep, such as “rsvp20” (UniProt ID: A4GZY3 and NCBI ID: NP_001087251.1), have an ambiguous support from the phylogenetic analysis. The same is true for the hedgehog/mole BSPs (Fig. 2a). The members of the three BSP subfamilies, which are interweaved either functionally and/or evolutionarily, will definitely spark a wider interest as model molecules in protein evolution and/or functional divergency studies.

In addition, proteins of the BSP subfamily are found in much higher concentrations in seminal plasma compared to the two other subfamilies (Fan et al. 2006; Lefebvre et al. 2007; Tian et al. 2009). Differences between BSPH1 and BSPH2 subfamilies are subtler. Whereas genes from BSP and BSPH1 subfamilies possess signal peptides cleaved in mature proteins, *in silico* analysis of BSPH2 genes reveals no putative cleavage sites for signal peptides (Fig. 3). This finding could suggest that BSPH2 proteins either possess a signal peptide conserved in their mature form or that they are transferred to sperm using a different mechanism, possibly via epididymosomes as has been observed for other epididymal proteins lacking signal peptide (Frenette et al. 2003). Another difference in BSPH2 proteins is the lack of the three tyrosine residues required for PC-binding (Fig. 3, red arrows). These missing residues have been shown to cause a decrease in the BSP ability to bind to PC liposomes and to prevent the promotion of sperm capacitation by BSPH2 proteins in mouse (Plante et al. 2014a). These many differences suggest that the proteins and genes of each subfamily have different roles in sperm functions as well as special specific characteristics.

Binding properties

Over the years, more than a dozen different interaction partners have been identified for proteins of the BSP superfamily (Table 1). Some of these interactions, such as those with calmodulin, high-density lipoproteins (HDL), apolipoprotein A-I (apoA-I), insulin-like growth factor II (IGF-II), phospholipase A₂ (PLA₂), annexins, phospholipids and glycosaminoglycans (GAG), have been ascribed to putative biological functions (Chandonnet et al. 1990; Desnoyers and Manjunath 1992, 1994; Ignatz et al. 2007; Manjunath et al. 1989, 1993, 1994b). Other interactions of BSP proteins such as with milk proteins (casein micelles, α -lactalbumin, β -lactoglobulin) and LDL have been associated with the protective effect of extenders used in sperm preservation (Bergeron et al. 2007; Manjunath et al. 2002) and is discussed later.

Gelatin

Gelatin, heat and/or acid denatured type I collagen, was the first binding partner identified for BSP proteins (Manjunath et al. 1987), and subsequently many other collagen types (I–V) were also shown to bind BSP proteins (Manjunath et al. 1988). This discovery was based on the fact that several Fn2-containing proteins were known to bind this ligand (Baker 1985; Cool et al. 1985; Engvall and Ruoslahti 1977). The binding of BSP proteins to gelatin is mostly due to hydrophobic interactions and could also involve ionic interactions since both urea and arginine can elute BSP proteins specifically from gelatin–agarose columns (Gasset et al. 1997; Manjunath et al. 1987). Dimerization of the proteins has also been suggested as being important for the binding (Manjunath et al. 1987; Plante et al. 2014a). Some residues, particularly tyrosine residues, have been shown to be essential for a strong gelatin-binding, but, most of all, a proper tridimensional structure is crucial for the binding as illustrated by the fact that the reduced or denatured Fn2 domains are unable to bind to gelatin (Banyai et al. 1990; Tordai and Patthy 1999). Despite the fact that all identified BSP proteins can interact with gelatin, this binding ability has not so far been linked to any known biological functions. It has, however, been extensively used to isolate and purify BSP proteins from the seminal plasma or seminal vesicle secretions of many mammals (Bergeron et al. 2005; Boisvert et al. 2004; Manjunath et al. 1987; Villemure et al. 2003).

GAG

Glycosaminoglycans such as heparin and dermatan sulfate (previously called chondroitin sulfate B) have been shown to bind BSP proteins (Chandonnet et al. 1990; Thérien et al. 2005). This interaction has been used on some occasions to purify BSP proteins from seminal plasma to obtain either better yields or higher purity (Lusignan et al. 2007). However, the study of the interaction of BSP proteins with GAG is of interest mainly because of the important role of GAG in diverse fertility processes, including capacitation, acrosome reaction and the interaction of sperm with the zona pellucida (ZP) (Coy et al. 2008; Parrish et al. 1989), and is discussed later.

Interaction of proteins with GAG is essentially attributed to electrostatic forces between clusters of basic amino acids in the proteins with the negative charges of GAG. Early studies suggested that consensus sequences found in bovine BSP proteins, such as B-B-X-B and B-B-B-X-X-B (B = basic residues and X = any residues), were possibly responsible for GAG binding (Cardin and Weintraub 1989; Chandonnet et al. 1990). Yet, even 20 years later, the identification of the sites responsible for formation of GAG–protein complexes is still an imprecise science if only based on the protein sequence (Munoz and Linhardt 2004). In the case of bovine BSP1, the

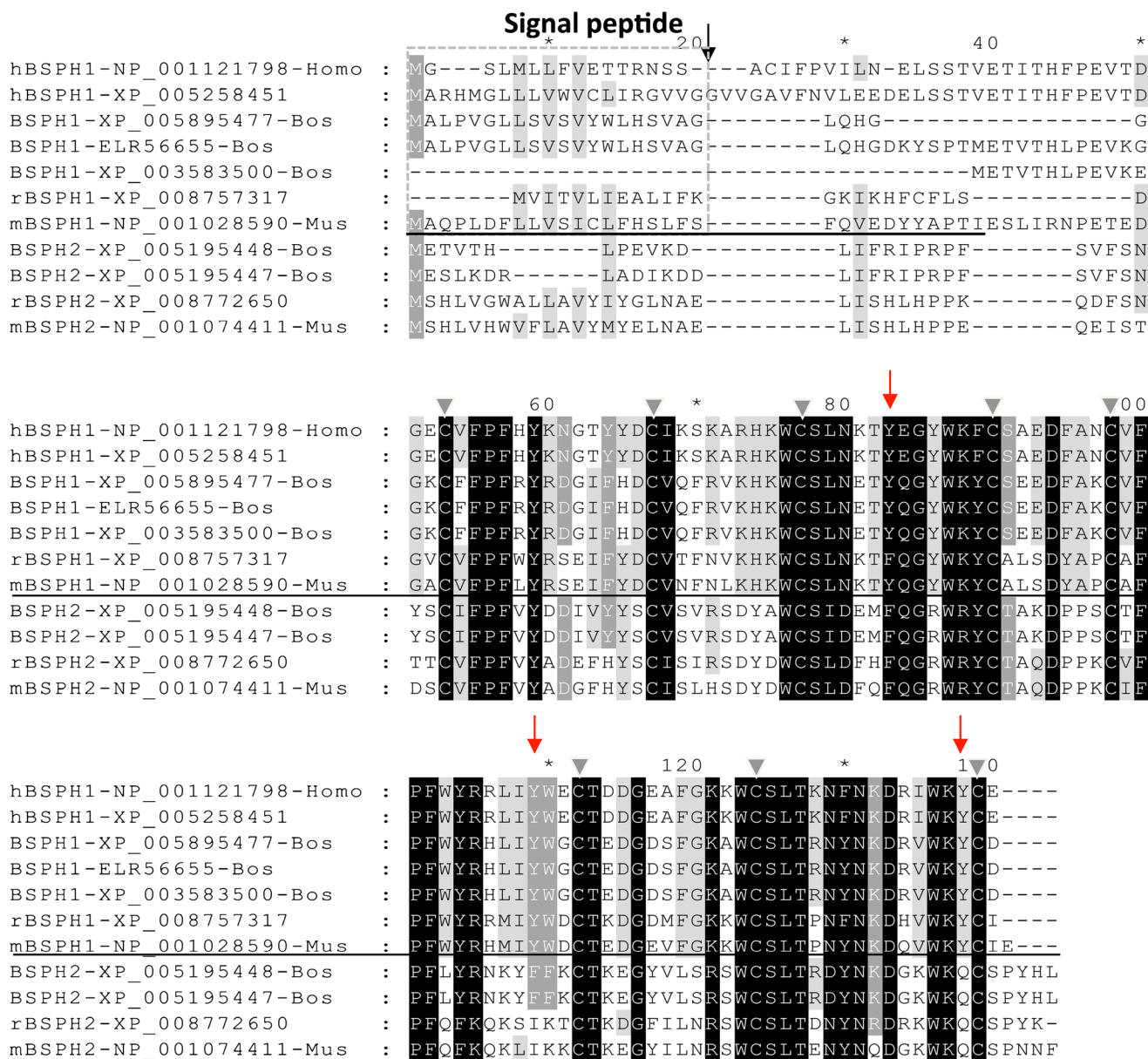


Fig. 3 Multiple sequence alignment of predicted amino acid sequences of BSPH1 and BSPH2 from human, bovine, rat and mouse. Amino acid sequences were aligned using ClustalW multiple alignment program (version 1.8). Identical amino acids are enclosed by *black* and conservative by *gray*, and less conservative by *light gray* boxes. The *line* below the alignment indicates the sequenced portion by 5-RACE as

presented in our previous publications. The predicted signal peptide is separated from the mature proteins by an *arrow*. The *downward arrow* indicates eight strictly conserved cysteine residues within the two tandem Fn2 domains. *Red arrows* indicate missing tyrosine residues in BSPH2 proteins. Each sequence name and its corresponding sequence GenBank accession number are indicated in front of each sequence in the alignment

resolution of its crystal structure does confirm the presence of conformational patches of basic amino acid responsible for the interaction with heparin (Wah et al. 2002). The heparin-binding property of BSP proteins can also be modulated by their aggregation state. In fact, only a fraction of the BSP proteins found in stallion seminal plasma, those forming aggregates, can bind to heparin, while the monomeric proteins cannot (Calvete et al. 1995b). This phenomenon was also proposed for bovine BSP1 proteins since isothermal titration calorimetry (ITC) analysis reveals the formation of an

oligomeric structure composed of 8–16 B.P. molecules per heparin molecule when these species interact (Sankhala et al. 2011a).

Phospholipids

The recognition with high specificity of the phosphocholine motif by BSP1 proteins is a key contribution of the protein affinity for phospholipid bilayers and membranes (reviewed in Swamy 2004). The two Fn2 domains mentioned previously

Table 1 BSP proteins' binding properties

		Gelatin	HDL	LDL	PC	Casein micelles	α -lactalbumin	β -lactoglobulin	Heparin	CSB
Bovine	BSP1	++	++	++	++	+	+	+	++	++
	BSP3	++	++	++	++	+	+	+	++	++
	BSP5	++	++	++	++	+	+	+	++	++
Boar	BSP1	+		++	++	+	+	+	++	++
Stallion	BSP1	++		++	++	–	++	++	++	
	BSP2	++		++	++	–	++	++	++	
Goat	14–15 kDa	++		++		++ ^a	–	++ ^a	+	
	20–22 kDa	++		+		++ ^a	–	++ ^a	++	
Bison	16 kDa	++		++					–	
	17 kDa	++		++					++	
	28 kDa	++		++					++	
Ram	15–16 kDa	++		++		+	+	+	++	
	22–24 kDa	++		++		+	+	+	++	
Human	BSPH1	+		++	++				++	–
Mouse	BSPH1	+	+		++				++	–
	BSPH2	+			–				++	–

– no interaction, + weak interaction, ++ strong interaction

^a Unpublished data

act as binding site for phosphocholine head group (Desnoyers and Manjunath 1992, 1993; Sticht et al. 1998; Wah et al. 2002). The structure of the protein crystallized in the presence of phosphorylcholine group, the soluble polar head group fragment of phosphatidylcholine, reveals the presence of the substrate in two binding sites located on the same face of the protein (Romero et al. 1997; Wah et al. 2002). The phosphorylcholine groups bind to the Fn2 domains through cation– π interactions between the quaternary ammonium group of the choline group and a protein tryptophan, as well as hydrogen bonding between hydroxyl groups of tyrosine and the phosphate group of the substrate (Wah et al. 2002). In addition to these specific interactions, it was proposed that hydrophobic interactions would also contribute to the binding of BSP1 proteins to bilayers. The analysis of the primary structure of BSP1 shows sections where apolar residues are mainly found (Seidah et al. 1987), providing hydrophobic pockets, as mentioned previously, susceptible to favor insertion of the protein in the apolar core of bilayers and membranes; the contribution of the hydrophobic interaction is supported by the fact that BSP1 association to membranes was shown to be an entropically-driven binding process (Anbazhagan et al. 2011). The association of BSP1 proteins with PC bilayers leads to an hypsochromic shift of the protein fluorescence (Anbazhagan et al. 2008; Muller et al. 1998), indicating a more apolar environment of the aromatic residues. The insertion of the protein in the external leaflet of PC bilayers was also inferred from the morphology changes observed upon binding to liposomes. Transmission electron microscopy, and fluorescence microscopy revealed that the presence of

BSP1 proteins leads to vesicle elongation, formation of lipid buds, pearl necklace-like structures, and even extrusion of long (>20 μ m) lipid nanotubes (Lafleur et al. 2010). These structures result from the increase of the external leaflet area of the bilayers relative to the inner one, an augmentation that is associated with the insertion of the protein. The capacity of BSP1 protein to bind PC bilayers is considerable; the saturation binding ratio is estimated to about 1 protein to 10–16 phospholipids (Anbazhagan et al. 2011; Gasset et al. 2000; Lassiseraye et al. 2008; Muller et al. 1998; Ramakrishnan et al. 2001), which actually corresponds to a protein monolayer with an area equivalent to the lipid layer.

The specific affinity of BSP1 proteins for PC compared to other phospholipid species has been assessed using different techniques. Using electron spin resonance (ESR) spectroscopy, it was found that the association of BSP1 proteins to DMPC bilayers leads to the immobilization of some spin-labelled lipids. The proportion of immobilized spin probes was dependent on the lipid polar head group with, from the most to the least affected: phosphatidic acid (PA) (pH 8.5) > PC \approx sphingomyelin (SM) \approx PA (pH 6.0) > phosphatidylglycerol (PG) \approx phosphatidylserine (PS) > phosphatidylethanolamine (PE) (Ramakrishnan et al. 2001). If one excludes PA, which is not significantly present in sperm membranes, these results indicate a preferential direct contact of PC and SM with the protein inserted in DMPC bilayers. The specificity for PC is also observed from the association constant of BSP1 protein to bilayers formed by a single phospholipid species and 20 mol % cholesterol, as determined from surface plasmon resonance (SPR) measurements. The inferred

affinity constant decreases in the following order: DMPC > DMPG > DMPA > DMPE (Thomas et al. 2003). Probing BSP1 protein association to lipid bilayers with the changes of protein fluorescence, it is inferred that BSP1 binding to PC membranes is hindered by the inclusion of PS or PE when the lipid/protein incubation ratio is low (Muller et al. 1998). All these results demonstrate the affinity of BSP1 proteins for phospholipid bilayers, highlight a specificity toward PC, and identify PE, the second most abundant phospholipid in sperm membranes (Nolan and Hammerstedt 1997) as a very poor binder.

The association of BSP1 proteins to lipid bilayers does not appear to significantly affect its secondary structure, the analysis of the Amide I band of the infrared spectra reporting a slight (Gasset et al. 2000) or no significant change (Lassiseraye et al. 2008). However, it provides stability to the protein as the thermal denaturation of the bound form is much more reduced compared to that of the free form. A similar effect is observed upon the binding of α -phosphorylcholine (Gasset et al. 1997), indicating that it is essentially the interactions between the ligand and the Fn2 domains that are responsible for this stabilization.

Phospholipid efflux

In addition to binding membranes, BSP proteins are also able to extract phospholipids and cholesterol from the outer membrane leaflet of red blood cells (Tannert et al. 2007a), fibroblasts (Moreau and Manjunath 1999), epididymal sperm (Thérien et al. 1998, 1999) and artificial membranes (Therrien et al. 2013). Early studies revealed that there is no correlation between the amount of BSP bound at the membrane surface and the lipid efflux induced (Moreau and Manjunath 2000). However, not all BSP can induce the efflux as efficiently. In bovine, the effect of BSP3 is much weaker than BSP1 or BSP5 (Thérien et al. 1999). The composition of the membrane can also affect the lipid efflux. ^{31}P NMR spectroscopy shows that lipid efflux induced by BSP1 proteins on model membranes is significantly higher for DMPC bilayers than for those formed with DMPG (Damai et al. 2010). The extraction of lipid is also slower in the presence of cholesterol (Tannert et al. 2007b).

Recently, a three-step lipid extraction mechanism was proposed for BSP proteins (Fig. 4). First, BSP proteins interact with and bind to the choline containing phospholipids in the membrane. Second, the proteins penetrate the external leaflet of the lipid membrane. Then, third, BSP proteins solubilize lipid patches from the membrane external leaflet without lipid specificity to form small particles containing about 15 lipids per protein (Therrien et al. 2013). Particles generated by the BSP-induced efflux from sperm membranes are proposed to have a diameter of 80 nm and are mainly composed of BSP

proteins, cholesterol and lipid bearing a choline moiety in a molar ratio of 0.05:1.21:1 (Moreau and Manjunath 1999).

Cholesterol

As much as the observations on the phospholipid-binding properties of BSP proteins provide a consistent picture, the possible interaction of BSP proteins with cholesterol, another major component of cell plasma membrane, is a subject of debate between experts in the field. Some observations suggest that addition of cholesterol to phospholipid bilayers increases the interaction of BSP proteins with phospholipids, and that BSP proteins can immobilize spin-labeled sterols in lipid bilayers (Greube et al. 2001; Swamy et al. 2002). However, it is unknown whether BSP proteins can interact directly with cholesterol or not. While it is clear that BSP proteins do not interact with immobilized cholesterol in ELISA assays or with cholesterol in solution (Desnoyers and Manjunath 1992; Moreau and Manjunath 1999; Muller et al. 2002), they could still interact with cholesterol inserted in phospholipid bilayers. Some data show that BSP1 decreases the mobility of sterols in membranes depending on the sterol side chain suggesting a direct interaction (Scolari et al. 2010). However, the decreased mobility of sterol could also be the result of a different effect of the sterol chains on bilayer properties modifying the interaction of BSP1 with the lipids. The hypothesis of a direct interaction is supported by the presence of cholesterol recognition amino acid consensus (CRAC) domains in BSP proteins (Scolari et al. 2010). Yet, independent studies have shown that the effect of BSP proteins on sterol mobility is dependent on the presence of PC and that replacement of PC by other phospholipids like PE or PS reduces the immobilization of sterols (Greube et al. 2001; Muller et al. 2002). These results suggest that the effect of BSP proteins on sterol could occur as a result of their overall effect on lipid membranes. Some more work is definitely needed to clarify this debate.

Semen preservation

Semen preservation can be used for several purposes including artificial insemination for animal breeding, conservation of endangered species, genetic improvement of domestic species or artificial reproductive technologies to solve human infertility problems (Barbas and Mascarenhas 2009). Mammalian sperm can usually be stored for a few days at 4 °C, or be frozen in liquid nitrogen (−196 °C) for several decades. In ram, semen frozen and stored for more than 27 years still retains its fertilizing ability (Salamon and Maxwell 2000). In order to preserve sperm for long periods, it is necessary to take precautions, as sperm cells are sensitive to changes in temperature, osmolarity and pH. Thus, sperm have to be diluted in extenders before they can be frozen.

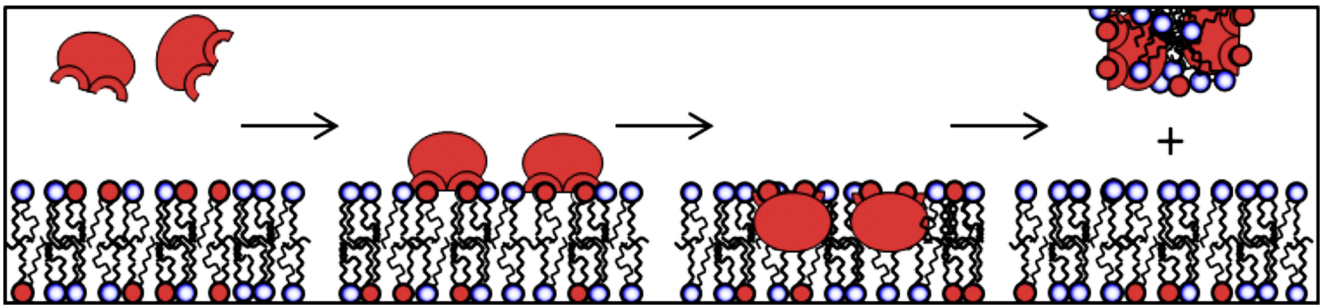


Fig. 4 Three-step mechanism for BSP1-induced extraction of lipids from sperm membranes. (Reproduced from Therrien et al. 2013)

Although sperm from distinct species are differently affected by freezing, all extenders have a similar basic composition regardless of the species. They are usually composed of a phosphate or Tris buffer supplemented with sugars, salts and antibiotics. To prevent cold-shock damage, extenders also contain non-invading cryoprotectants [egg yolk (EY), milk or soybean lecithin], as well as penetrating cryoprotectants like glycerol (Barbas and Mascarenhas 2009). However, the mechanism by which milk and EY can protect sperm remained elusive for several decades.

It has long been known that exposure of sperm to seminal plasma for extended periods is detrimental to sperm and that removal of the seminal plasma immediately after semen collection significantly improves sperm cryosurvival in most mammals (Chang 1957). This effect has been observed in semen from bulls, stallions, boars and rabbits to name a few (Eng and Oliphant 1978; Moore et al. 2005). The knowledge relative to the origin of this detrimental effect is, however, limited. BSP proteins, major components of the seminal plasma in many mammals, are detrimental for sperm preservation in liquid or frozen state (Manjunath et al. 2002). This is proposed to be due to the ability of BSP proteins to extract phospholipids and cholesterol from cell membranes in a time- and concentration-dependent manner, as continuous exposure of sperm to bovine BSP proteins can lead to the efflux of as much as 30–35 % of PC and cholesterol from sperm membranes (Manjunath et al. 2002; Thérien et al. 1998, 1999). This lipid efflux in turn weakens the sperm membrane and leads to increased capacitation rates leaving the cells sensitive to cold-shock (Thérien et al. 1998). To reduce this harmful effect, specific depletion of BSP proteins from seminal plasma has been proven to improve the motility, viability and acrosome integrity of sperm after thawing (Bergeron and Manjunath 2004; Manjunath et al. 2002; Srivastava et al. 2012, 2013). It is believed that milk and EY protect sperm in a similar manner, through sequestration of BSP proteins to prevent their detrimental action on sperm membranes (Bergeron et al. 2007; Bergeron and Manjunath 2006; Lusignan et al. 2011b; Manjunath et al. 2007). Interestingly, in line with the above hypothesis, in buffalo, the semen samples reacting badly to cryopreservation are known to contain higher levels of BSP1 than those that show a good response (Singh et al. 2014).

Sperm protection by EY

Early on, following the discovery of the detrimental effect of BSP proteins on sperm, it was demonstrated that BSP proteins interact with the low-density fraction of EY and more specifically with the low-density lipoproteins (LDL) (Manjunath et al. 2002). Binding of BSP proteins to LDL occurs very rapidly and has been observed for BSP proteins from bull, stallion, bison, ram and human (Bergeron et al. 2005; Boisvert et al. 2004; Lefebvre et al. 2009; Manjunath et al. 2002; Menard et al. 2003). The binding capacity of BSP proteins to LDL is also very high, one LDL particle binding up to 104 ± 5 BSP proteins (Lusignan et al. 2011a; Manjunath et al. 2002). Due to this high binding capacity, addition of EY to fresh ejaculate reduces the amount of free BSP proteins available for binding to sperm, which in turn diminishes phospholipid and cholesterol loss from sperm membranes (Bergeron et al. 2004). EY addition also keeps sperm motile, even after 24 h incubation at 4 °C (Bergeron et al. 2004). Based on these observations, it was thus proposed that, upon ejaculation, sperm are mixed with BSP proteins found in seminal plasma, which bind to sperm surface and induce cholesterol and phospholipid efflux leading to damaged sperm, less resistant to cold-shock. However, when ejaculates are mixed with EY, LDL sequesters most of the BSP proteins in seminal plasma, preventing membrane alterations and maintaining sperm viability and motility (Fig. 5) (Manjunath et al. 2007).

Sperm protection by milk

Milk extenders are usually prepared with heated skimmed milk or whole milk, both efficiently protecting sperm (Kakar and Ganguli 1978). The proposed mechanism of sperm protection by milk is analogous to that by EY but with a different BSP protein-capturing agent. As skimmed milk contains almost no lipid, sperm protection by milk cannot be due to lipid–protein interactions. In fact, many studies have shown that the protective constituent in milk is most likely micelles of caseins, the major proteins of milk. When milk devoid of caseins is used as extender, sperm viability and motility

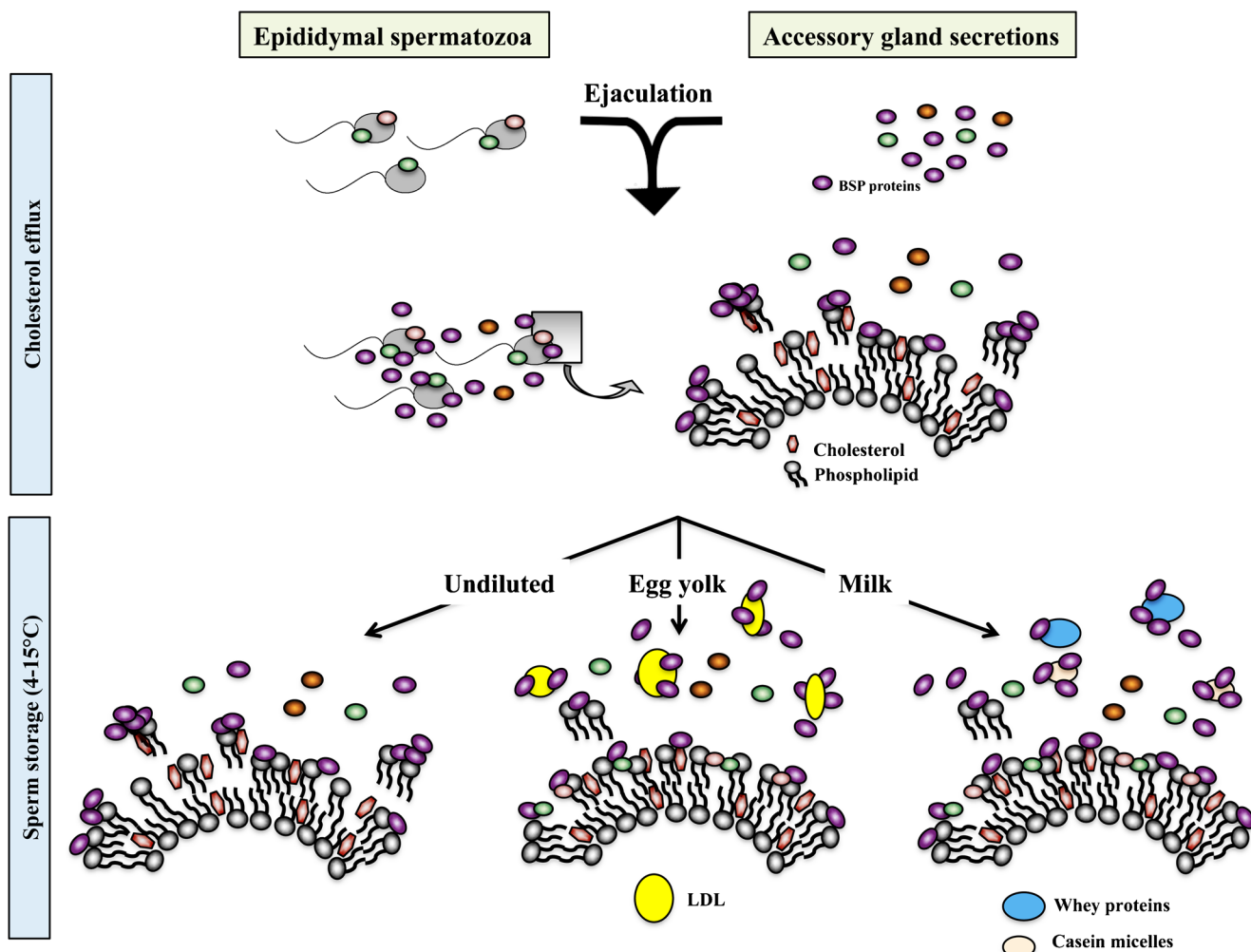


Fig. 5 Proposed mechanism of sperm protection by milk or egg yolk. (Adapted from Manjunath, et al. 2007)

decrease in a time-dependent manner, which is not the case with normal milk extenders (Bergeron et al. 2007). Extended analysis of the interaction of bovine BSP proteins with milk proteins revealed that all three BSP proteins interact with caseins. BSP1 and BSP5 can additionally interact with other milk proteins, namely α -lactalbumin and β -lactoglobulin (Lusignan et al. 2011b). Similar interactions are also observed for BSP proteins from boar, stallion, ram and goat (Plante et al. 2015).

The binding capacity of casein proteins to BSP proteins is, however, lower than that of LDL – there are about 4–5 bound BSP proteins per casein (Lusignan et al. 2011b). Analogous to sperm incubated with EY, bovine sperm incubated with milk have less BSP proteins bound to their surface following incubation at 4 °C and show lower loss of phospholipids and cholesterol (Bergeron et al. 2007). This has also been observed for goat sperm incubated with milk (Menezes et al., in preparation). Therefore, it is most probable that, when ejaculates are incubated with milk, the milk proteins in the extender sequester BSP proteins in a similar manner, as does LDL

from EY, preventing the BSP protein-induced extraction of phospholipids and cholesterol from sperm membranes, and as a consequence, protecting sperm (Fig. 5).

Are seminal plasma proteins detrimental or beneficial?

Recently, the results from a study showed that the concentration of BSP proteins on sperm surface increases following freeze/thaw cycles even in the presence of milk extenders (Ardon and Suarez 2013). This finding is not consistent with the results mentioned above indicating a reduction of free BSP proteins, available for sperm binding, in the presence of extenders. It should be pointed out that BSP proteins have a tendency to aggregate and that this aggregation increases following freezing and thawing (Ardon and Suarez 2013). Such large aggregates present in the seminal plasma following thawing are very hard to separate from sperm cells and may contribute to the apparent quantity of proteins on sperm surface. These data encourage more in-depth analysis to deepen our knowledge on the effect of freezing and thawing on

sperm–protein association. For example, immunofluorescence experiments present a powerful approach to specifically detect the proteins on the sperm surface.

As mentioned above, exposure of sperm to seminal plasma for extended periods has been shown to be generally detrimental to sperm in most species. However, reports for ram provide an inconsistent view. This situation comes from the fact that different approaches have been used to test the effect of seminal plasma proteins on ram sperm and therefore describe different phenomena. A series of works suggest that addition of seminal plasma protects sperm from cold-shock damage but without freezing (Barrios et al. 2000, 2005; Perez-Pe et al. 2001a). These studies (Ollero et al. 1997; Perez-Pe et al. 2001a) have examined the effect of the addition of seminal plasma proteins to ejaculated ram sperm washed by a dextran/swim-up method on cold-shock damage (rapid decrease of the temperature from 25 to 5 °C and then back to 25 °C). It was concluded that the presence of seminal plasma proteins, especially two Fn2-containing proteins of 14 and 20 kDa, respectively, identified as ram BSP1 and BSP5, improves sperm viability and repairs the cold-shock-induced damage of sperm membranes. This effect has been mostly attributed to the decapacitating role of BSP proteins and their stabilizing effect on the sperm membrane (Barrios et al. 2000). Other studies have focused their investigation on the effect of seminal plasma proteins on sperm protection following freezing and thawing in egg yolk extender. These studies indicate that addition of seminal plasma to sperm after freeze and thaw enhances sperm progressive motility (Bernardini et al. 2011; Dominguez et al. 2008; Leahy and de Graaf 2012). However, the situation is not as clear regarding the addition of seminal plasma before cooling–freezing. Some studies have indicated that seminal plasma helps the repair of sperm membranes and improves sperm viability and acrosome integrity (Bernardini et al. 2011; Leahy and de Graaf 2012). Conversely, other results show no positive effect on sperm viability, membrane integrity or acrosome status when seminal plasma is added to sperm prior to freezing (Dominguez et al. 2008). These results, in apparent contradiction, highlight the complexity of the effects of seminal plasma on sperm. As mentioned previously, the detrimental effect of seminal plasma and BSP proteins is concentration- and time-dependent (Manjunath et al. 2002; Moore et al. 2005; Thérien et al. 1999). In addition, different methodologies can put emphasis on different aspects of sperm preservation. For example, in ram, experiments are generally done using washed ejaculated sperm and supplemented with low concentrations of seminal plasma proteins (2.1 mg, equivalent to ~600 µg of ram BSP proteins per ml) or with ejaculated sperm in the presence of EY, which would sequester BSP proteins (Almadaly et al. 2015). Thus, under those conditions, the amount of free BSP protein is limited and may be below the threshold that is detrimental to sperm. Similar observations have been made in bovine where BSP

proteins are shown to have a positive effect on sperm preservation at low concentration, while they are clearly known to be detrimental to spermatozoa at high concentration (Almadaly et al. 2015). Some experiments in ram included short incubations of the sperm with BSP proteins (30–60 min). Similarly, in stallions, seminal plasma is known to have little effect on sperm viability and motility if sperm are frozen immediately after processing (Moore et al. 2005). It should be pointed out that, in bovine, the negative effect of BSP proteins on sperm is observed after prolonged incubation and at higher concentrations (mg quantities), while an hour-long incubation in the presence of low or moderate concentrations (µg quantities) of BSP1 or BSP3 is not sufficient to induce phospholipid efflux from sperm membrane (Thérien et al. 1999).

Methods used to isolate sperm from seminal plasma, although they do not affect membrane integrity in fresh semen, are known to affect the positive effect of seminal plasma on cold-shock damage (Perez-Pe et al. 2001b). This indicates that the positive effect observed does not solely reflect the effect of BSP proteins on cold-shock damage but is also linked to the *in vitro* manipulations performed during the experimentation. For example, it is possible that the methods used to isolate sperm modify the nature and the amount of proteins found on the sperm surface. Thus, addition of seminal plasma, before or after cold-shock, would simply restore what is missing and would then “protect” the sperm.

It appears that, at least in ram, the BSP proteins in low concentrations are essential for sperm functions. But, a recent proteomic study reveals that seminal plasma from ram ejaculates with high preservation ability at 15 °C did not have higher levels of BSPs than seminal plasma from ram ejaculates with low preservation ability (Soleilhavoup et al. 2014). After extended exposure of sperm to physiological concentration of seminal plasma following ejaculation, seminal plasma appears to be as detrimental to the cells as are BSP proteins from other species. These data bring a lot of valuable information regarding the importance of a minimal amount of sperm-bound BSP proteins acting as decapacitating factors in the stabilization of the membrane and in subsequent fertilization steps.

Biological functions

BSP proteins are mostly known for their implication in sperm capacitation, but are involved in many more steps of fertilization. Other functions ascribed to BSP proteins include molecular chaperones, markers of cell death, roles in sperm motility and viability, in the formation of the oviductal sperm reservoir, in the regulation of cell volume and in the interaction between sperm and oocytes.

Sperm motility

Sperm acquire their motility during their transit through the epididymis and are kept in a quiescent state while they are stored in the cauda part of the epididymis. Following ejaculation, their motility increases to allow their transport to the site of fertilization. Most of the sperm proteins implicated in motility are usually found over the midpiece of the flagellum of the cells. BSP proteins have also been found to bind to these two sperm structures in many species, namely bulls, humans, mice, rabbits and rams, suggesting their implication in sperm motility (Barrios et al. 2005; Manjunath et al. 1994a; Nixon et al. 2008; Plante et al. 2012, 2014a, 2014b).

Bull BSP1, which is in very high concentrations in the seminal plasma following ejaculation, has been shown to increase the motility of sperm in a dose-dependent manner, probably via the activation and/or enhancement of the activity of Ca^{2+} -ATPase (Sanchez-Luengo et al. 2004). Plasma membrane Ca^{2+} -ATPase-4 (PMCA4) has been proposed as the probable target of BSP1. In vitro studies clearly show that BSP1 can enhance the activity of PMCA4 in sperm extract, but a direct interaction between BSP1 and PMCA4 has never been reported in vivo (Post et al. 2010; Triphan et al. 2007). The hypothesis of a direct interaction is rather unlikely because BSP1 binds to the sperm surface whereas PMCA4 is located underneath the plasma membrane (Aumuller et al. 1988; Post et al. 2010). Interestingly, some results suggest an opposite effect of BSP proteins, since BSP5 was shown to be more abundant in ram with lower sperm motility (Rodrigues et al. 2013). Work is still needed to understand the implication of BSP proteins in sperm motility and how the differences in BSP protein concentrations in seminal plasma can affect motility.

Sperm capacitation

Capacitation is a sperm maturation step essential for fertilization of an oocyte that takes place in the oviduct, in the female genital tract (Austin 1952; Chang 1951). It is mainly characterized by modifications in the composition of sperm plasma membrane, the increase in intracellular pH, the increase in calcium permeability and the activation of several signaling pathways leading to increased tyrosine phosphorylation (de Lamirande et al. 1997; Go and Wolf 1983; Langlais and Roberts 1985; Visconti and Kopf 1998). To induce sperm capacitation, some extrinsic factors are necessary, including bicarbonate, calcium, and sterol acceptors like albumin or HDL (Bailey 2010). The exact order in which events leading to sperm capacitation occur is still unclear, but it is generally accepted that it is first induced by a cholesterol and phospholipid efflux from sperm membranes, probably emancipated from the membrane by albumin or HDL (Cross 2000). This efflux from sperm membranes is usually accompanied by a

decrease in the membrane cholesterol/phospholipid ratio and closely followed by increases in intracellular calcium concentration and in pH, leading to the activation of several signaling pathways necessary for the subsequent steps of fertilization (Visconti et al. 1999). These activated pathways include the protein kinase A (PKA) pathway, the protein kinase C (PKC) pathway, the extracellular signal-regulated kinase (ERK) pathway and the phosphatidylinositol-3-kinase (PI3K)/Akt pathway (Breitbart et al. 1992; de Lamirande and Gagnon 2002; de Lamirande et al. 1997; Fisher et al. 1998; Luconi et al. 1998; Nauc et al. 2004; Visconti et al. 1995).

In some species, GAG such as heparin can also promote sperm capacitation as efficiently as HDL or albumin, but by a different mechanism. The first mechanism proposed for heparin-induced capacitation, a mechanism still accepted nowadays, suggests that, during epididymal maturation and upon ejaculation, some heparin-binding proteins are added to sperm and act as decapacitating factors to prevent premature capacitation. When sperm are thereafter exposed to heparin or heparin-like GAG, decapacitating factors are released from the sperm membrane leading to the increase in intracellular calcium and the activation of the pathways (Parrish et al. 1988). Some of these heparin-binding decapacitating factors are now known to be members of the BSP superfamily.

The possible implication of BSP proteins in sperm capacitation was suggested very soon following their discovery in bovine seminal plasma (Chandonnet et al. 1990; Manjunath et al. 1989), when they were found to interact with HDL and heparin, and was validated a few years later (Thérien et al. 1995). BSP proteins are found to promote sperm capacitation in bulls, boars, humans and mice (Lusignan et al. 2007; Plante et al. 2014b; Plante et al. 2012; Thérien et al. 1995, 1998). This effect is also proposed in stallions, goats and rams (Barrios et al. 2005; Calvete et al. 1995a; Serrano et al. 2013; Villemure et al. 2003). In bovine, BSP proteins cannot induce capacitation by themselves unless sperm are exposed to high concentrations of proteins for prolonged periods, conditions that do not occur in vivo (Thérien et al. 1999). They do, however, potentiate the capacitation induced by heparin or by HDL (Thérien et al. 1995, 1998, 2001). In order to induce capacitation, both Fn2 domains of the proteins are necessary (Moreau et al. 1998; Jois et al. 2015). As mentioned earlier, mechanisms of heparin- and HDL-induced capacitation are very different and, in that regard, the role of BSP proteins in both mechanisms is equally different.

HDL-induced capacitation

The implication of BSP proteins in HDL-induced capacitation is believed to be restricted to the first step of the process, namely the perturbation of the sperm plasma membrane, as both HDL and BSP proteins are individually able to extract cholesterol and phospholipids from sperm membrane (Gasset

et al. 1997; Moreau et al. 1999; Moreau and Manjunath 1999; Thérien et al. 1997, 1999). Although pre-incubation of bovine epididymal sperm with BSP proteins, prior to their incubation with HDL, slightly increases the percentage of cholesterol extracted from membranes, it has no direct effect on the ability of HDL to extract cholesterol from the sperm membrane (Thérien et al. 1998). Nevertheless, it can significantly accelerate the HDL-induced capacitation (Thérien et al. 2001). Taking this into consideration, in addition to the fact that antibodies specific against bovine BSP proteins are unable to inhibit HDL-induced capacitation, it is proposed that BSP proteins in bovine are implicated in, but not essential for, HDL-induced capacitation (Lane et al. 1999). This statement, however, cannot be extended to all mammals as recent data in mice show that fragments of antibodies against BSPH1 can completely inhibit HDL-induced capacitation (Plante and Manjunath 2015b).

Two hypotheses have been proposed to explain the implication of BSP proteins in HDL-induced capacitation. The first one suggests that BSP proteins could be implicated in the docking of HDL via apoA-I to the cell membrane and would act more as receptors for HDL (Manjunath et al. 1989). However, studies have shown that, even though some BSP proteins remain on the sperm surface following capacitation, exposure of sperm to oviductal fluid containing HDL and GAG displaces most of them from the membrane (Einspanier et al. 1999; Souza et al. 2008). The second and favored hypothesis proposes that, once BSP proteins are bound to the sperm surface, they cannot promote cholesterol or phospholipid efflux unless they interact with acceptors such as free BSP proteins or HDL (Moreau and Manjunath 2000). Therefore, upon ejaculation, BSP proteins coat the surface of sperm cells and the binding is rapidly saturated (Moreau and Manjunath 2000). Due to their high concentration in the seminal plasma, the remaining of the BSP proteins are free, act as acceptors and induce a first cholesterol efflux, which ends when sperm pass through the cervical mucus in the female genital tract and free BSPs are removed. BSP proteins bound to the sperm surface can thereafter stabilize the sperm membrane until sperm reach the oviduct (Fig. 6a). Once sperm enter the site of fertilization and are exposed to follicular and oviductal fluid, HDL interacts with BSP proteins and removes them from the membrane, creating a second cholesterol and phospholipid efflux leading to the initiation of sperm capacitation (Fig. 6b) (Manjunath and Thérien 2002). As mentioned earlier, HDL does not require the presence of BSP proteins to remove cholesterol and phospholipids from the sperm membrane. The mechanism by which BSP proteins and HDL induce efflux from the membranes and the composition of the particles extracted by the two species are completely different (Moreau and Manjunath 1999, 2000). Thus, it is probable that HDL induces capacitation acting alone as well as through an interaction with BSP proteins; the first way favoring

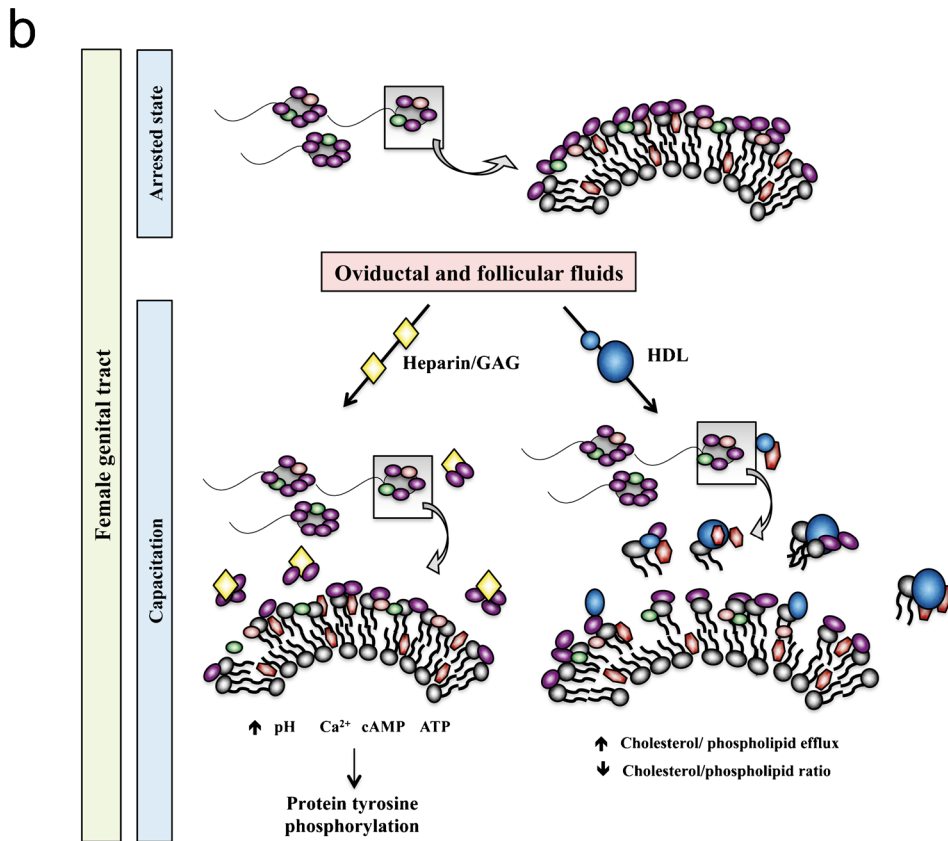
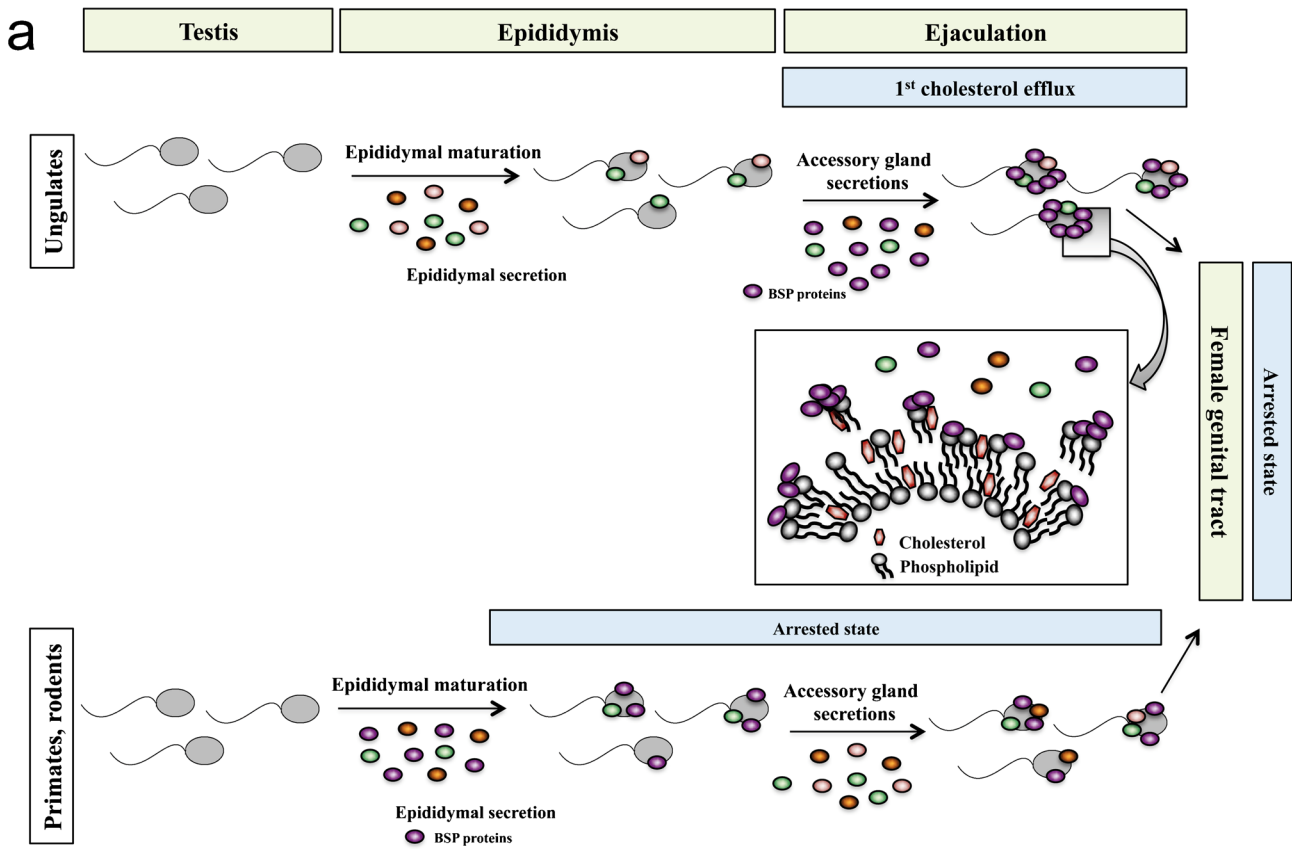
extraction of cholesterol and the second, extraction of phospholipid to accelerate and maximize the capacitation of sperm.

Heparin-induced capacitation

The implication of heparin in sperm capacitation cannot be extended to all mammals, as heparin can directly induce acrosome reaction in some species. The role of BSP proteins in heparin-induced capacitation has been less studied than its implication in HDL-induced capacitation, but the presence of BSP proteins seems to be essential for this process as it can be inhibited in a dose-dependent manner by antigen-binding fragments (fabs) against BSP proteins (Lane et al. 1999). In bovine species, heparin is believed to promote sperm capacitation by removing heparin-binding decapacitating factors from the sperm surface (Parrish et al. 1988). Thus, as opposed to HDL-induced capacitation, heparin does not affect the lipid composition of the membrane but rather initiates the activation of signaling cascades resulting in increased tyrosine phosphorylation (Lane et al. 1999). The exact mechanism by which the interaction of heparin and BSP proteins leads to the activation of signaling pathways and the exact pathways activated are at the moment unknown, but some hypotheses can be proposed. For example, addition of BSP1 can inhibit the activity of the PKC involved in signaling events leading to the induction of acrosome reaction (Yu et al. 2003). A direct interaction between both proteins has never been observed in vivo, but it is possible that the presence of BSP proteins on the sperm surface prevents premature acrosome reaction by inhibition of this kinase, and, that upon contact with heparin, this inhibitory effect is lifted.

Bovine BSP proteins have also been shown to bind to calmodulin, a protein regulating several cell processes in a calcium-dependent manner (Manjunath et al. 1993). As mentioned above, calcium is one of the key components for sperm maturation. Capacitation and acrosome reaction are both calcium-dependent processes. Calmodulin is implicated in both processes in sperm and in several intracellular signaling events as well as in sperm motility (Bendahmane et al. 2001;

Fig. 6 Proposed mechanism for BSP protein-induced capacitation. **a** BSP proteins are added to sperm surface differently in ungulates when compared to primates and rodents. In the latter, BSP proteins are added to sperm during epididymal maturation where they coat the sperm surface to stabilize the membrane and prevent premature capacitation. In ungulates, BSP proteins are added upon ejaculation and promote a first efflux of cholesterol and phospholipids. Once sperm enter the female genital tract, free BSP proteins are removed and BSP proteins bound to sperm surface stabilize the membrane. **b** Then, mechanisms of capacitation in the female genital tract could be similar for all mammals. Once sperm enter the oviduct, they come into contact with GAG and HDL from follicular and oviductal fluid. Both GAG and HDL interact with BSP proteins and remove them from the sperm surface, activating signaling pathways and promoting cholesterol and phospholipid efflux respectively leading to capacitation. (Adapted from Manjunath, et al. 2007; Thérien et al. 2001, 2005)



Fournier et al. 2003; Lasko et al. 2012; Si and Olds-Clarke 2000). Interestingly, in bovine, several studies have shown that the presence of heparin in concentrations sufficient to induce capacitation decreases the binding of calmodulin to sperm, but also induces a decrease in the interaction between calmodulin and other sperm surface proteins (Leclerc and Goupil 2000; Leclerc et al. 1989, 1990).

A third interaction that could be implicated in the BSP/heparin-induced capacitation is the interaction between BSP proteins and PLA₂. PLA₂ is an enzyme, which, when activated, can cleave phospholipids into fatty acids and lyso-phospholipids, an important step in the priming of sperm for the acrosome reaction. Not only is this enzyme calcium-dependent and regulated in part by calmodulin but it can also be stimulated and inhibited in a concentration-dependent manner by BSP proteins (Leclerc et al. 1989; Manjunath et al. 1994b; Soubeyrand et al. 1998). In high concentrations, such as upon ejaculation when sperm membranes are saturated with BSP proteins, activity of PLA₂ is inhibited. However, in the presence of low concentrations of BSP proteins, following capacitation for example, activity of PLA₂ can be increased more than 100-fold.

Finally, BSP proteins in bovine have also been shown to interact with IGF-II, a protein found in bovine seminal plasma that has been shown to increase sperm motility (Desnoyers and Manjunath 1994; Henricks et al. 1998). Although not much is known about the role of IGF-II in sperm functions or in capacitation, IGF-I, a protein structurally similar, is shown to be involved in sperm capacitation and acrosome reaction in boar, rabbit and human (Miah et al. 2008; Minelli et al. 2001; Naz and Padman 1999; Sanchez-Luengo et al. 2005). In boar, IGF-I can increase sperm progressive motility, as well as the induction rate of capacitation and of acrosome reaction in a cholesterol efflux-independent mechanism (Miah et al. 2008). More work is clearly needed to fully understand the implication of the IGFs in sperm capacitation, but, at this point, it appears that their role could be modulated through their interaction with BSP proteins.

Based on these observations, it is possible to propose that, upon ejaculation, BSP proteins not only coat the surface of the sperm membrane to stabilize the phospholipids but also to inhibit key enzymes and prevent premature capacitation and acrosome reaction. When exposed to heparin or heparin-like GAG found in follicular and oviductal fluids, BSP proteins are removed from the membrane, disrupting BSP interaction with sperm proteins such as calmodulin and PLA₂, leading downstream to the activation of kinases like PKC and to subsequent steps of fertilization like the acrosome reaction of sperm (Fig. 6b).

BSP proteins from epididymis vs seminal vesicles

Until very recently, most of the data accumulated on sperm capacitation were obtained with BSP proteins expressed by

seminal vesicles and added in high concentrations to sperm upon ejaculation. Subsequent analysis of BSPH1 proteins from mice and human revealed that these proteins, found in very low concentrations in seminal plasma, are already bound to the sperm surface in the epididymis and are able to promote sperm capacitation as efficiently as proteins expressed by seminal vesicles (Plante et al. 2012, 2014b). Therefore, the effect of BSP proteins as decapacitating factors and later on the promotion of sperm capacitation could be the same regardless of the concentration of the proteins and/or the site of their expression (Fig. 6a). However, the concentration of BSP proteins is most probably directly linked to the site of their expression. In ungulates, proteins expressed in the seminal vesicles are added to sperm only upon ejaculation and are removed quickly once sperm go through the cervical mucus, leaving little time for BSP proteins to coat the sperm surface or to have any detrimental effect on the cells. In contrast, epididymal maturation is a long process lasting approximately 10 days, which leaves plenty of time for BSP proteins to interact with the sperm membrane. For this reason, it is likely that the expression of low concentrations of BSP proteins in this organ is necessary to prevent cholesterol and phospholipid efflux produced by the presence of large amounts of BSP proteins. The resulting effect of binding of BSP proteins to sperm in both cases is the same: stabilization of the membrane and prevention of premature capacitation and AR.

BSPH2 proteins

For a long time, all BSP proteins studied were found to have the ability to bind to PC and to induce sperm capacitation. However, recently, mouse BSPH2 was found unable to do so (Plante et al. 2014a). These results were supported by simulations revealing that the interaction of BSPH2 with PC is weaker than that of murine BSPH1 or bovine BSP proteins (Fig. 3). As BSP proteins can induce capacitation partly based on their ability to extract lipids from membranes, the weak binding of BSPH2 to PC is the likely cause of its inability to promote sperm capacitation. Interestingly, all the BSPH2 proteins identified so far (mouse, rat and bovine) lack the same three tyrosine residues, suggesting that members of the BSPH2 subfamily could all be unable to promote capacitation.

Formation of the sperm reservoir

For fertilization to occur, each step has to be well orchestrated and must occur at the right moment and in the right place. In cattle and in other mammals including mice, hamsters, pigs and horses, once sperm enter the isthmus following ejaculation, they can attach themselves to the oviductal epithelium to form a sperm reservoir. This direct contact with the epithelium is known to extend the viability of sperm, to preserve their fertilizing ability and to regulate capacitation and

hyperactivation, in order to provide sperm supply when ovulation occurs (Chian et al. 1995; Dobrinski et al. 1997; Pollard et al. 1991).

Characterization of the interaction between sperm and oviductal epithelium explant revealed that the binding is due to the interaction of BSP proteins on the sperm surface with fucose-containing annexins on the epithelial surface (Ignatz et al. 2001, 2007; Suarez et al. 1998). Accordingly, binding of bovine epididymal sperm or bovine capacitated sperm to oviductal explants is significantly lower than ejaculated spermatozoa (Gwathmey et al. 2003; Hung and Suarez 2012). In addition to being responsible for the binding of sperm to the oviductal epithelium, BSP proteins are also responsible for prolonging the motile lifespan of sperm (Gwathmey et al. 2003, 2006).

Upon ejaculation, BSP proteins bind to sperm and excess BSP proteins are removed following the passage through cervical mucus. Once in the oviduct, sperm bind to the epithelium via an interaction of BSP proteins with the fucose groups of annexins. This binding stabilizes sperm membranes, prevents premature capacitation and prolongs sperm survival possibly via the inhibition of calmodulin and PLA₂ (Suarez 2007). Following ovulation, factors such as GAG are released from the follicular fluid into the oviduct, interact with both annexins and BSP proteins, and detach sperm from the reservoir to allow subsequent steps of fertilization, such as capacitation, acrosome reaction and fertilization (Hung and Suarez 2012; Ignatz et al. 2007). Although only tested in bovine, the implication of BSP proteins in the formation of the sperm reservoir could possibly be extended to boar, as protein aggregates from boar seminal plasma interacting with the oviductal epithelium have also been shown to contain the porcine BSP1 (Liberda et al. 2006).

Sperm–egg interaction

Once sperm are capacitated, some BSP proteins still remain bound to the sperm surface, a fact that has led to suggestions of a putative role for these proteins in sperm–egg interactions (Manjunath et al. 1988; Muino-Blanco et al. 2008). Even though this role has not been clearly demonstrated, several experiments seem to indicate that BSP proteins could be involved in the interaction between sperm and the ZP. The ZP is a layer adjacent to the plasma membrane of the oocyte, which is composed of three or four glycoproteins depending on the species. In bovine, these proteins possess saccharide chains mainly composed of mannose, a binding partner of BSP proteins (Liberda et al. 2002). Some preliminary, unpublished data also seem to indicate that BSP proteins in bovine can bind to isolated ZP and that incubation of sperm with antibodies against BSP1 can inhibit the binding of sperm to oocytes (Turmo et al. 2009). Similar observations have been made in

rabbit where incubation of anti-BSP1 in a dose-dependent manner decreased fertilization rates (Nixon et al. 2008).

Other functions

Two other functions, namely a role in cell volume regulation and as molecular chaperones, have recently been suggested for BSP proteins. The first role has been proposed after it was demonstrated that cauda sperm from bull incubated in the presence of BSP1 were more swollen in response to hypotonic stress than sperm incubated in the absence of BSP1 (Sahin et al. 2009). However, no further developments have been made following this publication. The second role implicating BSP proteins as molecular chaperone has been tested with BSP proteins from bulls and stallions (Sankhala et al. 2012; Sankhala and Swamy 2010). BSP from both species were shown to possess a chaperone-like activity *in vitro*, preventing the unfolding and aggregation of other proteins, protecting proteins against stress conditions, and directing the native proteins to an active conformation. Based on these observations, it was suggested that BSP1 could help the fertilization process by directing aggregated or misfolded proteins of the seminal plasma and/or bound to the sperm surface toward a functionally active conformation (Sankhala et al. 2011b; Sankhala and Swamy 2010). Whether BSP proteins act as chaperone *in vivo* under physiological conditions still remains to be determined.

Are BSP proteins fertility markers?

Due to their implication in so many steps of fertilization, BSP proteins are considered by many as potential markers for predicting the fertility of cattle or for determining the sensitivity of samples to cryopreservation. For example, in ram, sperm with higher resistance to cryopreservation were shown to have higher concentrations of BSP proteins in their seminal plasma (Dominguez et al. 2008; Leahy et al. 2010). In bull, however, the presence of bovine BSP1 on the acrosome region of sperm following ejaculation was suggested to be a marker for sperm sensitivity to cryopreservation (D'Amours et al. 2012). Furthermore, in bovine, all three BSP proteins appear to be expressed in higher concentrations in bulls with low fertility (D'Amours et al. 2010; Lessard et al. 2011; Roncoletta et al. 2006). While some reports suggest that BSP protein concentration has a strong negative correlation with sperm viability (D'Amours et al. 2010), other studies suggest instead an optimal protein concentration and that a concentration too low or too high would be detrimental to fertility (Moura et al. 2006). Finally, an ubiquitinated form of BSP5 has also recently been reported to accumulate on the surface of defective ejaculated sperm, while it was not found on normal sperm (Odhiambo et al. 2014). BSP proteins may be markers of fertility or cryosensitivity in sperm from farm animals, but the available

data are insufficient to establish a good correlation between BSP proteins/binding patterns and fertility.

Conclusion

After more than three decades of studies on BSP proteins, it is now possible to state that these proteins, ubiquitous amongst mammals, are important in many aspects of fertility. They can be both beneficial and detrimental to sperm and can be considered as potential markers of fertility. Their study is essential not only to better understand their roles in sperm motility, viability, the formation of the oviductal sperm reservoir, the regulation of cell volume, the sperm–oocyte interaction and in capacitation but also to determine the effect of seminal plasma on sperm during semen preservation. This knowledge is required to improve the efficiency of sperm conservation and to develop novel extenders free of products of animal sources. Although an extensive amount of work has been done in elucidating the mysteries of BSP proteins, much work is still ahead in order to clarify the role of BSP proteins in diverse biological functions. Studies must also focus on the clarification of the possible dual effects of BSP proteins in semen preservation. Finally, more research is clearly needed to fully understand the way that these proteins are expressed and regulated and the different subtleties of the BSP subfamilies, most specifically BSPH2 proteins.

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