Implication of the human Binder of SPerm Homolog I (BSPH1) protein in capacitation

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ABSTRACT: Binder of SPerm (BSP) proteins are a family of proteins expressed exclusively in the male reproductive tract (seminal vesicles or epididymis) of several mammalian species. They are known to promote capacitation, a sperm maturation step essential for fertilization. Our recent studies have shown that in human, the Binder of SPerm Homolog I (BSPH1) is expressed solely in epididymal tissues. The goal of the current study was to characterize BSPH1 and evaluate its effect on different sperm functions. A human recombinant BSPH1 (rec-BSPH1) was produced, purified and refolded. Rec-BSPH1 was found to share many characteristics with other members of the BSP superfamily, as it was able to bind gelatin and heparin as well as capacitate sperm. Rec-BSPH1 had no effect on sperm acrosome reaction or any sperm motility parameters. Native BSPH1 was localized on the equatorial segment, post-acrosomal segment and neck of ejaculated human sperm. Rec-BSPH1, following incubation with washed ejaculated human sperm, exhibited binding patterns similar to the native protein. These results show that the human epididymal BSPH1 shares many biochemical and functional characteristics with BSP proteins secreted by seminal vesicles of ungulates, and behaves similarly to its murine epididymal orthologue BSPH1. This study of human BSPH1 brings us one step closer to understanding the importance of this protein in male fertility.

Key words: Binder of SPerm proteins / epididymal protein / fibronectin type II domains / human sperm capacitation / recombinant protein

Introduction

The Binder of SPerm (BSP) proteins (previously called Bovine Seminal Plasma proteins) are a superfamily of proteins highly conserved among mammals. They are characterized by their structure composed of a variable N-terminus domain followed by two fibronectin type II (Fn2) domains arranged in tandem, and a variable C-terminus tail (Manjunath et al., 2009). BSP proteins were first identified in the bovine (BSP1, BSP3 and BSP5 previously called BSP-A1/A2, BSP-A3 and BSP-30K, respectively) where they represent ≈ 60% of the total proteins in seminal plasma. Homologs of these proteins, secreted by seminal vesicles, have also been identified in the seminal plasma of stallion, boar, goat, bison, ram and buffalo (Calvete et al., 1995a, 1997; Menard et al., 2003; Villemure et al., 2003; Boisvert et al., 2004; Bergeron et al., 2005; Harshan et al., 2009). Several roles in sperm functions have been suggested for the bovine BSP proteins, including the mediation of sperm binding to the oviductal epithelium, the ability to prolong sperm survival and motility in the oviduct and a chaperone-like activity (Gwathmey et al., 2003; SanKhala and Swamy, 2010). However, the main function associated with BSP proteins is their ability to promote sperm capacitation (Therien et al., 1998, 2005; Lane et al., 1999; Lusignan et al., 2007).

Capacitation is one of the key steps of sperm maturation that takes place in the female genital tract following ejaculation and is necessary for sperm to acquire the ability to fertilize an oocyte (Austin, 1951; Chang, 1951). The molecular mechanisms underlying sperm capacitation are not well understood but this step of the maturation is usually characterized by changes in the lipid composition of the sperm plasma membrane, increase in intracellular pH, increased permeability to ions such as calcium and increased tyrosine phosphorylation of a group of signaling proteins (Austin, 1951; Chang, 1951; Go and Wolf, 1983; Langlais and Roberts, 1985; Suarez, 1996; de Lamirande et al., 1999; Visconti and Kopf, 1998). Some factors can induce sperm capacitation in vitro. The most commonly used capacitation factor in vitro is bovine serum albumin (BSA). Similar to bovine BSP proteins, BSA can remove
cholesterol from the sperm plasma membrane, but also induces sperm protein phosphorylation and calcium influx (Salicioni et al., 2007; Xia and Ren, 2009).

Unique features of the BSP Fn2 domains have been used to find new BSP-homologous sequences in the genome of different species. Two BSP-homologous genes in mouse (BspH1, BspH2; accession numbers DQ227498, DQ227499) and one in human (BSPH1; accession number DQ227497) were identified (Fan et al., 2006). As opposed to BSP proteins in other species, human and murine proteins are exclusively expressed in the epididymis (Lefebvre et al., 2007). Recently, recombinant murine Binder of Sperm Homolog 1 (BSPH1) and human recombinant BSPH1 (rec-BSPH1) proteins were produced and shown to share many biochemical properties with BSP proteins from ungulates (Lefebvre et al., 2009; Plante et al., 2012). Human rec-BSPH1 was shown to interact with phosphorylcholine (PC), egg yolk low-density lipoproteins (LDLs) and human ejaculated sperm (Lefebvre et al., 2009). Murine BSPH1 is orthologous to human BSPH1. They share ~56% identity and 78% similarity (Lefebvre et al., 2007). Murine rec-BSPH1 was shown to interact with gelatin (denatured type I collagen), heparin, PC and epididymal sperm. It was also able to promote sperm capacitation (Plante et al., 2012).

The aim of the current study was to further characterize the human rec-BSPH1 protein. To do so, a recombinant protein was expressed, purified and refolded on column. The protein was then used to test its binding properties to various ligands and test its effect on sperm capacitation, acrosome reaction (AR) and sperm motility. This study brings us one step closer to elucidating the role of BSPH1 in human sperm functions.

Materials and Methods

Protein expression and purification

The recombinant protein was expressed as previously described (Lefebvre et al., 2009). Briefly, Origami B(DE3)pLysS cells (Novagen, EMD Biosciences, La Jolla, CA, USA) transformed with pET32a vectors (Novagen) containing the cDNA sequence of BSPH1 were inoculated in 250 ml of Luria–Bertani medium containing 100 µg/ml of ampicillin (Sigma-Aldrich, Oakville, ON, Canada). Bacteria were incubated at 37°C with shaking at 200 rpm until O.D600nm reached 0.6–0.8. To induce the expression, isopropyl β-D-1-thiogalactopyranoside (IPTG; Invitrogen, Carlsbad, CA, USA) was added to the cell culture to a final concentration of 1 mM and cells were incubated at 15°C, 200 rpm for 16 h. Following the induction, cells were harvested by centrifugation at 6000g for 10 min at 4°C.

Purification method was modified from Plante et al. (2012). Cell pellets were resuspended in B-Per bacterial protein extraction reagent (Pierce, Rockford, IL, USA) as described by the manufacturer and subjected to sonication (seven cycles of 10 s on ice). One volume of 4 × binding buffer (2 M NaCl, 80 mM Tris–HCl, 20 mM imidazole pH 7.9) was added to the cell lysate. Urea was added to a final concentration of 6 M, the volume was adjusted with water to four times the initial volume and the cell extract was centrifuged at 4°C at 20 000g for 30 min. The supernatant was filtered through a 1 µm filter and loaded on a column (1 cm × 15 cm) containing 5 ml of His-Bind resin (Novagen) charged with Ni2+ and equilibrated with 1 × binding buffer containing 6 M urea at a flow rate of 24 ml/h. The column was washed with 5 bed volumes of 1 × binding buffer containing 6 M urea and 5 bed volumes of washing buffer (500 mM NaCl, 20 mM Tris–HCl, 80 mM imidazole, 6 M urea, pH 7.9). Bound proteins were then refolded on-column by use of a decreasing urea gradient (6–0 M) over 16 h in 1 × binding buffer (total volume 250 ml). Finally, the refolded proteins were eluted with three successive elution buffers containing different imidazole concentrations (500 mM NaCl, 20 mM Tris–HCl, pH 7.9 containing 150, 200 and 400 mM imidazole, respectively). Based on O.D280, similar quantities of all samples were precipitated with trichloroacetic acid (TCA; final concentration 15%) and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting. Proteins were also extracted from polyacrylamide gel and analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS; Havlis et al., 2003). The rec-BSPH1 protein is produced using a pET32a vector, which adds three tags to the protein. A thioredoxin (Trx) tag to help the solubility of the protein, a (His) 6-tag and an S-tag both to aid protein purification. To make sure that these tags are not responsible for the effects observed, the Trx-His-S tag expressed without BSPH1 was used as a control. Trx-His-S control was prepared in the same way using Origami B(DE3)pLysS containing empty pET32a vectors and were purified by immobilized metal ion affinity chromatography (IMAC) in the absence of urea.

Protein electrophoresis and western blotting

SDS–PAGE was performed according to the method of Laemmli (1970) in 15% polyacrylamide gels using the Mini-Protein Tetra Cell apparatus from Bio-Rad (Mississauga, ON, Canada). Gels were either stained with Coomassie Brilliant Blue R-250 (Bio-Rad), or transferred electrophoretically to Immobilon-P Polyvinylidene difluoride (PVDF) membranes (Millipore, Nepean, ON, Canada). Immunodetection was performed using either His-Probe mouse monoclonal antibodies (Santa Cruz, Santa Cruz, CA, USA), affinity-purified antibodies monoclonal antibodies (Santa Cruz, Santa Cruz, CA, USA), affinity-purified antibodies against synthetic peptides corresponding to the last 15 C-terminal amino acids (SLTKNFNKDRIWYKCE) of the deduced sequence of BSPH1 (anti-16mer) or affinity-purified antibodies against (His)6-tagged rec-BSPH1 (anti-BSPH1) at a dilution of 1:1000 (Lefebvre et al., 2009). Goat anti-mouse IgG (1:3000) or goat anti-rabbit IgG (1:10 000) were used as secondary antibodies (Bio-Rad). The bands were revealed using a chemiluminescence reagent (ECL kit, PerkinElmer, Boston, MA, USA) and a Fuji LAS-3000 image analyzer (Fujifilm; Stamford, CT, USA).

Affinity chromatography

All operations were carried out at 4°C. Heparin–Sepharose CL-6B resin was purchased from Amersham Biosciences (Baie d’Urfe, QC, Canada). The coupling of gelatin to agarose beads and of chondroitin sulfate B (CSB; Sigma-Aldrich) to Affi-gel 15 were performed as previously described (Manjunath et al., 1987; Therien et al., 2005; Plante et al., 2012). For each experiment, 5 ml of resin was packed in a column (1 cm × 15 cm) and equilibrated with 25 mM Tris–HCl buffer, pH 7.9 (TB). 500 µg of rec-BSPH1 dissolved in 5 ml of TB were applied to the column at a flow rate of 2 ml/h and the flow rate was stopped for 30 min. Flow rate was increased to 25 ml/h and the unbound material was then washed from the column with TB. Bound proteins were finally eluted with TB containing 1 M NaCl for heparin–Sepharose and CSB–agarose columns or with TB containing 8 M urea and 100 mM choline chloride for gelatin–agarose column. The protein fractions from each peak were pooled and ~3 µg of protein from each peak were precipitated with TCA and analyzed by SDS–PAGE. For quality control, alcohol precipitates of bovine seminal plasma proteins were run similarly on each column.

Homology modeling and molecular docking analyses

Putative 3D structure of human BSPH1 and murine BSPH1 were predicted via an automated comparative protein modeling server (swiss-model; http://www.expasy.ch) with the optimized mode using the coordinates of BSPI.
Preparation of sperm

All the experiments with sperm were approved by the ethics committee of the Maisonneuve-Rosemont hospital. Semen specimens were obtained by masturbation from healthy volunteers after a minimum of 2 days of sexual abstinence. After liquefaction, sperm were isolated from the seminal plasma by a four layers percoll gradient centrifugation (2 ml fractions each of 20, 40 and 65% and 0.1 ml of 95% percoll) made isotonic in HEPES-buffered saline as described by Lachance et al. (2007). The highly motile population was recovered in the 65–95% interface and within the 95% percoll fraction, pooled, diluted in a modified Biggers–Whitten–Whittingham medium without BSA (BWWV; 10 mM HEPES, 94.6 mM NaCl, 4.8 mM KCl, 1.7 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25.1 mM NaHCO3, 5.6 mM D-glucose, 21.6 mM Na-lactate, 0.25 mM Na-pyruvate, 0.4 μg/ml phenol red, pH 7.4; Biggers et al., 1971; Lachance et al., 2007) and their concentration was determined by sperm class analyzer (SCA) system (Microptic, Barcelona, Spain).

Capacitation and AR assays

For capacitation studies, sperm were incubated at 20 × 10^6 cells/ml for 4 h at 37°C, 5% CO2 in modified BWW medium alone or in the presence of different concentrations of rec-BSPH1 (0–60 μg/ml) or without protein, or with control Trx-His-S (32 μg/ml) or with fatty acid free BSA (3 mg/ml; capacitation condition). At the end of incubation, sperm samples were divided into two aliquots and calcium ionophore A23187 (Sigma-Aldrich) was added (10 μM final diluted in dimethylsulfoxide, DMSO) to one of the two aliquots to induce the AR while the other served as a control for spontaneous AR (same amount of DMSO was added to control). Both tubes were incubated for an additional 30 min, after which sperm were washed with phosphate-buffered saline (PBS), centrifuged at 500 g for 5 min, resuspended in absolute methanol for 30 min on ice, smeared on slides and air-dried. Each slide was incubated at room temperature for 30 min in the dark with a droplet of 1:20 diluted in dimethylsulfoxide, DMSO) to one of the two aliquots to induce the AR while the other served as a control for spontaneous AR (same amount of DMSO was added to control). Both tubes were incubated for an additional 30 min, after which sperm were washed with phosphate-buffered saline (PBS), centrifuged at 500 g for 5 min, resuspended in absolute methanol for 30 min on ice, smeared on slides and air-dried. Each slide was incubated at room temperature for 30 min in the dark with a droplet of 75 μg/ml of Piam satum agglutinin conjugated to fluorescein isothiocyanate (PSA-FITC, Sigma) diluted in PBS. Slides were rinsed with distilled water and mounted with DABCO 1.5% (90% glycerol containing 1.5% (w/v) 1,4-diazobicyclo(2,2,2)-octane) as an anti-bleaching agent. The acrosomal status was next evaluated according to the fluorescent pattern observed upon binding to the PSA-FITC under UV illumination (Cross et al., 1986). A minimum of 400 cells was counted for each treatment performed in duplicate.

Tyrosine phosphorylation

Tyrosine phosphorylation experiments were done as previously described (Lachance et al., 2007). After 4 h of incubation, sperm were washed with 1 ml PBS and centrifuged at 500g for 5 min. Sperm proteins were solubilized in sample buffer, processed for electrophoresis and transferred onto PVDF membrane. Membranes were blocked with 5% (w/v) skimmed milk in TB containing 0.02% Tween-20. Membrane was incubated with a monoclonal anti-phosphotyrosine antibody (4G10; Upstate Biotechnology Inc., Lake Placid, NY, USA) for 1 h at room temperature, washed and incubated with a GAM-HRP for 1 h. Western blot was revealed using ECL kit. To ensure that equal amount of proteins were loaded in each well of the gel, the same membrane was re-probed with a monoclonal anti-alpha-tubulin antibody (B5-1-2; Sigma) using the same procedure. The FluorChem® Q system (Alpha Innotech Corporation) was used for signal detection and densitometric analysis were performed using the AlphaView™ Q software. The phosphotyrosine protein/alpha-tubulin ratio was then calculated.

Evaluation of sperm motility parameters

During the 4 h capacitation studies, the effects of rec-BSPH1 proteins and controls on sperm motility were assessed using the SCA system with the following standard set-up parameters: number of frames to analyze, 25; number of frames/s, 25; straightness (STR) threshold, 80%; cell size range (low), 2; cell size range (high), 60; sperm concentration/ml, ≥ 20 × 10^6 cell/ml and forward motility, ≥ 50%. At different times, an aliquot of sperm suspension was loaded into a prewarmed 20-μm Leja Chamber (Somagen Diagnostic, Edmonton, Alberta, Canada) onto the heated stage of an Nikon Eclipse 50i microscope equipped with a positive phase contrast objective (×10) (Nikon Canada Inc. Instruments, Mississauga, ON, Canada) connected to the SCA system. All motility parameters were evaluated for at least eight randomly selected fields for each sample so that a minimum of 1000 sperm per condition were assessed. To evaluate sperm hyperactivation, following parameters were used: curvilinear velocity >75 μm/s, linearity <40% and amplitude of lateral head displacement >3.5 (Boue et al., 1994; Ferlin et al., 2012).

Immunofluorescence

Sperm were incubated at 20 × 10^6 cells/ml for 1 and 4 h (5% CO2, 37°C) in modified BWW medium in the presence of 30 μg/ml rec-BSPH1, 16 μg/ml Trx-His-S or without any added protein. Following incubations, sperm were washed three times for 5 min at 400 g and resuspended in 200 μl of PBS. Sperm were fixed by the addition of 200 μl of paraformaldehyde 4% for 15 min at room temperature and washed another three times. Sperm were then resuspended in 100 μl of PBS and 10 μl were smeared and dried on poly-l-lysine coated microscopic slides (Fisher Scientific, Ottawa, ON, Canada). Sperm were permeabilized with ice-cold methanol for 15 min, washed three times with PBS and blocked for 1 h at room temperature in PBS containing 1% BSA. Slides were then incubated overnight at 4°C with a mix of anti-BSPH1 antibodies (1:400)/anti-α-mer (1:400), mouse His-probe antibodies (1:400) or Normal Rabbit Serum IgG (NRS-IgG) as control (1:200) in PBS containing 0.1% BSA. The slides were washed three times with PBS to remove excess antibodies and incubated 1 h at room temperature with FITC-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) at dilution 1:400 in PBS—0.1% BSA. All slides were finally washed three times with PBS and mounted with DABCO 1.5%. Sperm were observed under a fluorescence microscope (Zeiss Axio Imager).

Statistical analysis

The data were expressed as mean ± SEM for AR and motility parameters. Differences among treatments were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test using GraphPad Instat (version 3.05). A P-value of <0.05 was considered significant.

Results

Protein expression and purification

Proteins were expressed in Escherichia coli Origami B(DE3)pLysS using a pET32a vector as detailed previously (Lefebvre et al., 2009). The purification method was modified to include refolding of the rec-BSPH1 protein on-column. After elution with different concentrations of imidazole, proteins were found in every fraction as seen on Fig. 1A. Analysis of the different fractions on 15% polyacrylamide gel (Fig. 1B) revealed the presence of a band at 32 kDa, the molecular weight of rec-BSPH1.
in the three fractions corresponding to elution with different imidazole concentrations (E1–E3). To confirm the identity of rec-BSPH1, fractions were also analyzed by western blot. The 32 kDa band was strongly recognized by anti-16mer and anti-BSPH1 antibodies. Previous method of purification for rec-BSPH1 did not include a refolding step. Analysis of these proteins under non-reducing conditions revealed the presence of proteins in highly aggregated complexes (Fig. 1C, lane 1). However, following on-column refolding, a bigger proportion of proteins were found as monomers, dimers and tetramers (Fig. 1C, lane 2). LC–MS/MS analysis of the 32 kDa band confirmed the identity of BSPH1 protein (Fig. 1D). Since fraction E1, corresponding to elution with buffer containing 150 mM of imidazole was not as pure as E2 and E3, corresponding to elution using 200 and 400 mM imidazole, respectively, only fractions E2 and E3 (pooled) were used for subsequent experiments.

**Binding to gelatin and glycosaminoglycans**

The capacity of rec-BSPH1 to bind to gelatin, heparin or CSB, known ligands of the BSP superfamily, was tested by affinity chromatography. As shown on Fig. 2A and B, when rec-BSPH1 was passed on a gelatin–agarose affinity chromatography column, ≏ 25–30% of the total proteins was retained and eluted in the presence of 100 mM choline chloride and 8.0 M urea. The recombinant protein bound more strongly to heparin as ≏ 95% was retained on the heparin–sepharose column and was eluted with 1 M NaCl (Fig. 2C and D). Contrary to the heparin–sepharose column, rec-BSPH1 was found in majority (≏ 98%) in the unabsorbed fraction when passed on a CSB–agarose affinity column (Fig. 2E and F). Binding of bovine BSP1 protein was also tested on the three resins used for this study as control (data not shown). BSP1 bound strongly to all three resins.
Homology modeling and molecular docking analyses

The 3D structure of human BSPH1 was found to be very similar to mouse BSPH1 as well as the previously well-characterized bovine BSP1 (Fig. 3A). Molecular docking analyses of bovine BSP1 and human BSPH1 with heparin heptasaccharide and CSB as ligands revealed differences in binding sites between BSP1 and BSPH1. Heparin heptasaccharide was found to bind to the groove between the two Fn2 domains of BSPH1 with an affinity of 2 $6.2$ kcal/mol (Fig. 3B), but appeared to bind to the groove extended to the Fn2 domain A of BSP1 with an affinity of 2 $4.5$ kcal/mol (Fig. 3C). A bigger difference was observed for the binding of CSB as the gelatin and glycosaminoglycan (GAG) appeared to bind in the front side groove between the Fn2 domains in BSPH1 with an affinity of 2 $6.9$ kcal/mol (Fig. 3D), but appeared to bind to BSP1 on the top surface between the two Fn2 domains with an affinity of 2 $5.2$ kcal/mol (Fig. 3E).

Immunolocalization of native and rec-BSPH1 on ejaculated sperm

Localization of the native BSPH1 and rec-BSPH1 on sperm surface was tested after 1 and 4 h incubation in BWW media at 37°C under 5% CO2 (Fig. 4). For sperm incubated for 1 h, alone or in presence of rec-BSPH1, a signal could be observed on the sperm head over the equatorial and post-acrosomal segment. The signal was stronger over the equatorial segment. The signal observed for sperm incubated in the presence of rec-BSPH1 was stronger than the signal of the native BSPH1 on sperm incubated alone. Immunofluorescence was also performed immediately after Percoll wash to evaluate native BSPH1 localization.
prior to the incubations. Results were similar in intensity and localization to those obtained following 1 h incubation (not shown). After 4 h incubation, fluorescence was still visible over the equatorial segment and post-acrosomal region, but a stronger signal appeared over the neck of the sperm for both sperm incubated alone and sperm incubated in the presence of rec-BSPH1. Immunodetection was tested on both permeabilized and non-permeabilized sperm (data not shown). Similar results were obtained in both instances, but the signals were stronger following permeabilization with methanol.

When sperm were incubated with rec-BSPH1 and NRS-IgGs were used as first antibodies for immunofluorescence, no signal was observed after 1 or 4 h incubation. Similarly, no signal was observed when ejaculated sperm were incubated with Trx-His-S and His-probe antibodies were used for immunofluorescence.

**Figure 3** Homology modeling and molecular docking of the BSPH1 with its potential ligands: heparin heptasaccharide and CSB. (A) Ribbon representative of the superposed view of the homology model of human BSPH1 (hBSPH1) with the homology model of murine BSPH1 (mBSPH1) and the structures of BSP1 chain A and chain B (PDB: 1H8P). (B–E) Surface view of hBSPH1 (B, D) and bovine BSP1 (C, E) proteins in 20% transparent docking with its ligands heparin heptasaccharide in stick colored in brown or CSB in stick colored in green, respectively. The arrow in Fn2-A of BSPH1 and Fn2-B of BSP1 indicates the favorable PC-binding properties in each protein.
Capacitation assay

Capacitation was assessed by the ability of sperm to undergo AR induced by calcium ionophore A23187 (Fig. 5). Without the addition of rec-BSPH1 or BSA, in the absence of A23187, the basal level of AR was 6 \pm 1% after 4.5 h. The addition of rec-BSPH1, Trx-His-S or BSA did not affect the level of spontaneous AR. When sperm were incubated 4.5 h with 3 mg/ml of BSA and then 30 min with A23187, the level of AR reached up to 31 \pm 3%. In counterpart, the addition of rec-BSPH1 instead of BSA for 4.5 h caused a dose-dependent increase in the level of AR reaching a value of 28 \pm 2% when 60 mg/ml of protein was added. A significant increase in the level of AR was reached using a minimum of 10 \mu g/ml of recombinant protein. The addition of 32 \mu g/ml of Trx-His-S to the media did not change the level of sperm AR.

The ability of rec-BSPH1 to induce the phosphorylation of the tyrosine residues of signaling proteins was also evaluated during the capacitation studies (Fig. 6). Results showed that the addition of rec-BSPH1 in different concentration had no impact on the phosphorylation of p105, but showed a significant decrease in the tyrosine phosphorylation level of p81 in the presence of 30 \mu g/ml rec-BSPH1. However, the addition of 16 \mu g/ml of Trx-His-S caused the same decrease in tyrosine phosphorylation.

Effect of rec-BSPH1 on sperm motility

Total motility, progressive motility and hyperactivation of ejaculated sperm were assessed using a SCA system at 2 and 4 h incubation in BWW alone, in the presence of rec-BSPH1, BSA or Trx-His-S. After the percoll gradient (time 0 h; white bars), 86 \pm 3% of the sperm collected were motile (Fig. 7A). After 2 h (grey bars) and 4 h (black bars) incubation in BWW medium alone, motility did not change significantly (83 \pm 3 and 76 \pm 2%, respectively). At different conditions tested total motility did not change significantly.

The progressive motility level at the beginning of the incubation was 65 \pm 3% (Fig. 7B). After 2 h incubation in BWW medium alone, the level of progressive motility remained similar at 67 \pm 3% and slightly decreased after 4 h incubation to 56 \pm 3%. Addition of rec-BSPH1, Trx-His-S or BSA did not change the levels of progressive motility significantly after 2 or 4 h.

Finally, at the beginning of the incubation, the level of hyperactivation was 2.9 \pm 0.4% (Fig. 7C). After 2 and 4 h incubation, levels of hyperactivation without any added proteins reached 6.1 \pm 1.7 and 4.6 \pm 0.9%, respectively. The addition of different rec-BSPH1 concentrations, 3 mg/ml BSA or of 32 \mu g/ml of Trx-His-S did not cause any significant increase of the level of hyperactivation when compared with sperm.

Figure 4 Immunodetection of native and rec-BSPH1 on ejaculated sperm. Ejaculated sperm separated on percoll gradient were incubated alone (no protein) or, in the presence of 30 \mu g/ml rec-BSPH1 or in the presence of 16 \mu g/ml Trx-His-S for 1 or 4 h. For immunofluorescence (IF), slides were incubated with a mixture of anti-16mer and anti-BSPH1 antibodies (anti-BSPH1 mix) at dilution of 1:400 each (first and second columns), His-probe antibodies at dilution of 1:400 (third column) or incubated with IgG purified from normal rabbit serum (1:200, last column). All slides were then treated with goat anti-mouse/anti-rabbit FITC-conjugated IgG. Original magnification \times 630. DIC, differential interference contrast.
incubated in BWWW medium alone. Therefore, addition of rec-BSPH1, BSA or Trx-His-S did not affect any of the three motility parameters tested after 2 and 4 h incubation in vitro.

**Discussion**

**Expression and purification**

Human and murine BSP proteins are found in minute amounts in seminal plasma, making it difficult to purify them in sufficient quantities to perform functional studies. The presence of four disulfide bridges in the two Fn2 domains makes it challenging to produce recombinant proteins in bacteria. In early experiments, producing recombinant BSP proteins in *E. coli* resulted in the production of insoluble, misfolded proteins that accumulated in inclusion bodies (Lefebvre et al., 2009). The use of *E. coli* Origami B(DE3)pLysS cells with a pET32a expression vector is often used to produce proteins with disulfide bridges successfully (Prinz et al., 1997; Bessette et al., 1999; Peisley and Gooley, 2007; Lefebvre et al., 2009; Plante et al., 2012).

Such an expression system combined with conventional IMAC purification lead to the production of proteins that were misfolded, aggregated and hard to solubilize (Fig. 1C, lane 1). Furthermore, these proteins could not promote sperm capacitation (not shown). To resolve these issues, purification by IMAC combined with on-column protein refolding using a decreasing urea gradient was used. This resulted in the production of over 95% pure proteins with good yield and lower levels of oligomerization (Fig. 1C, lane 2). Lower levels of oligomerization, such as the presence of dimers and tetrads, has been observed for native BSPH1 (Kumar et al., 2008) as well as in BSP proteins of other species (Manjunath and Sairam, 1987; Calvete et al., 1995a, b; Gasset et al., 1997; Bergeron et al., 2005). In addition, it has been demonstrated that some of the binding properties of bovine, boar and stallion BSP proteins depend on their ability to oligomerize and adopt specific quaternary structures (Calvete et al., 1995a, b, 1999).

**Binding properties**

Proteins of the BSP superfamily share many biochemical and binding characteristics. Among these are binding to GAGs, such as heparin and CSB, high-density lipoproteins (HDLs), LDL, choline phospholipids and gelatin (Manjunath et al., 1987; Chandonnet et al., 1990; Desnoyers and Manjunath, 1992; Sanz et al., 1993; Calvete et al., 1995a, b;
Roles of human rec-BSPH1 in sperm functions

Therien et al., 1998, 2005; Lane et al., 1999; Manjunath et al., 2002; Menard et al., 2003; Villemure et al., 2003; Bergeron et al., 2004, 2005; Boisvert et al., 2004). Many studies have tried to elucidate the structure responsible for these different interactions. Binding to gelatin has been attributed to the presence of the two Fn2 domains while binding to heparin and CSB has been attributed to the interaction of basic amino acids with the negatively charged GAGs (Manjunath et al., 1987; Hileman et al., 1998; Salek-Ardakani et al., 2000). Some studies have also identified essential amino acid(s) responsible for the binding to different ligands (Collier et al., 1992; Calvete et al., 1999; Tordai and Patthy, 1999; Wah et al., 2002).

When murine and human BSPH1 sequences were aligned with sequences of bovine BSP proteins (Fig. 8), it was evident that most residues essential for gelatin-binding (green) or PC binding (purple) are highly conserved. Binding studies performed using rec-BSPH1 are in accordance with those observations as rec-BSPH1 has previously been shown to bind PC liposomes as well as human ejaculated sperm (Lefebvre et al., 2009) and, in the current study, was found to partially bind to gelatin. The exact reason for the reduced binding to gelatin is unknown, but it is possible that a particular oligomerization state is required for the binding. However, the ability to bind gelatin is not linked with the role of BSP proteins in capacitation. The recombinant bovine BSP5 protein (Jois et al., manuscript in preparation) as well as murine rec-BSPH1 (Plante et al., 2012) do not bind gelatin strongly, but are able to promote sperm capacitation.

As mentioned above, GAG-binding is attributed to the presence of basic amino acids in BSP proteins. When sequences are aligned, it is possible to see a variation in the amount and organization of basic amino acid in different BSP proteins which is likely responsible for the surface charges that influence the ligand bindings. In the affinity chromatography experiments, rec-BSPH1 was found to bind to heparin but not CSB. In silico analyses were performed to better understand the interaction of BSP proteins with heparin as well as GAGs. To do so, the homology models of human and murine BSPH1 were deduced from the crystal structure of bovine BSP1. BSP1 does not possess any C-terminal tail and its crystal structure lacks the N-terminal 21 amino acids due to the disordered structure of this domain (Wah et al., 2002; Jois and Manjunath, 2010). For this reason, ligand-binding comparison to the two Fn2 domains of human BSPH1 and bovine BSP1 was analyzed. Comparison of the heparin binding to bovine BSP1 and to human BSPH1 reveals that the docking position of ligands in both proteins is consistent with our previous reports, where the Fn2-A domain of BSP1 is favored for the binding to heparin and GAGs and the Fn2-B domain is favored more for the conserved binding to PC (Fan et al., 2006). These binding preferences differ in BSPH1 as the Fn2-A domain is more favored for the binding to PC, which make the Fn2-A domain less likely to bind other ligand such as GAGs (Plante et al., 2014). Considering the different structural requirements for different ligand bindings, and spatial arrangements of physico-chemical properties in the binding sites in terms of protein oligomerization, we believe that molecular docking could be envisaged to interpret properly protein structure–function relationships (i.e. heparin binding to both protein surface; in the groove between the two Fn2 domains for BSPH1, or in the groove extended to the Fn2-A for BSP1). The major difference for the binding of CSB to BSPH1 and BSP1 reside in the fact that the CSB binding on BSPH1 was not located on the surface as shown for BSP1. This discrepancy could explain why rec-BSPH1 could not interact with CSB in the binding experiments. Based on those results, it appears that the interaction of the BSP proteins with GAGs is more complex than simply the presence of basic amino acid in consensus sequences or not. Molecular docking using tetramer protein instead of using monomer as receptor may be helpful in this aspect.

**Immunolocalization**

In a previous study, rec-BSPH1 was shown to bind ejaculated sperm, but the exact binding pattern on sperm surface was unknown (Lefebvre et al., 2009). In the current study, after 1 h incubation, both native BSPH1 and rec-BSPH1 were found over equatorial segment and post-acrosomal region. After 4 h a strong signal was detected over the neck as well.

**Figure 7** Effect of rec-BSPH1 on human sperm motility parameters. Ejaculated sperm were separated on percoll gradient, incubated in the presence of different concentrations of rec-BSPH1, Trx-His-S or BSA for 4 h and analyzed using a SCA system. Total motility (A), progressive motility (B) and hyperactivation (C) were assessed at the beginning of the incubation (0 h, white bars), after 2 h (grey bars) and 4 h (black bars). Data are presented as the mean ± SEM of six independent experiments. Differences compared with control (sperm alone) were analyzed by one-way ANOVA followed by Bonferroni post hoc test.

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The appearance of the signal over the neck did not seem to be linked with capacitation or AR of sperm as sperm incubated 4 h in the absence of rec-BSPH1 or BSA did not have increased levels of capacitated sperm, but had fluorescence over the neck of the sperm. Proteins binding to the equatorial segment and post-acrosomal region are thought to be implicated in capacitation as well as in sperm–egg interaction/fusion, whereas proteins binding to the neck of the sperm are more implicated in sperm motility (Lasserre et al., 2001). The presence of BSPH1 protein over the neck after 4 h of incubation could indicate a role in motility, but no changes in motility parameters were observed during that time period (Fig. 7). Results shown in Fig. 4 indicate that the signal of the native BSPH1 on human sperm is weaker especially when compared with the signal of BSP1 on bull sperm (Manjunath et al., 1994). This could be due to their relative concentrations in male reproductive tract. BSPH1 is found in low concentration and BSP1 represents up to 60% ($\approx 50 \text{ mg/ml}$) of the seminal plasma protein content. Therefore, the quantity of BSPH1 detected on human sperm surface is lower than what is observed on bull sperm.

All binding experiments using human rec-BSPH1 have been performed using ejaculated sperm due to the difficulties in obtaining human live epididymal sperm. However, BSPH1 is a protein expressed in the epididymis. Since many modifications can occur following ejaculation, binding of rec-BSPH1 to murine epididymal sperm was tested. As it was demonstrated for the mouse rec-BSPH1 (Plante et al., 2012), human rec-BSPH1 was able to bind to murine epididymal sperm (not shown). Human and mouse BSPH1 are orthologous proteins and possess very similar characteristics. Our studies in mice showed that murine rec-BSPH1 can bind to epididymal sperm and promote sperm capacitation similarly to what is observed for human rec-BSPH1 with ejaculated sperm (Plante et al., 2012). Based on those observations, it is possible that rec-BSPH1 would act on human epididymal sperm like they do on ejaculated sperm.

### Sperm capacitation

BSP proteins have been shown to promote sperm capacitation in bovine, boar and, more recently, it was shown that murine rec-BSPH1 can also promote sperm capacitation (Therien et al., 1995; Lusignan et al., 2007; Plante et al., 2012). Although the mechanism of sperm capacitation is not well understood, we know that, in bovine, BSP proteins can interact with choline phospholipids on the sperm membrane and cause a phospholipid and cholesterol efflux that can induce sperm capacitation. HDL as well as free BSP (unbound to sperm) appear to act as acceptors to induce the lipid efflux (Therien et al., 1998; Manjunath et al., 2007). Results in the present study showed that 4 h incubation with rec-BSPH1 could induce a dose-dependent increase in sperm capacitation. The effect of rec-BSPH1 on human sperm capacitation is similar to that observed with the murine rec-BSPH1 as in both cases a minimum 10 mg/ml of recombinant proteins cause significant increase in sperm capacitation (Plante et al., 2012).

It has been shown that sphingomyelin (choline containing phospholipid) can influence the rate of capacitation by controlling the loss of sterols and that sterol loss could represent the initial event of capacitation (Cross, 2000). It was also shown that HDL present in human uterine/follicular fluid is implicated in the lipid efflux from sperm membrane (Martinez and Morros, 1996). It has been demonstrated in bulls that, to promote sperm capacitation, BSP proteins require HDL or free (bound) BSP to act as acceptors and induce lipid efflux (Therien et al., 1995, 1998, 2001; Manjunath et al., 2007). In a similar manner, in this study, when low concentrations are used (lower than 10 mg/ml), proteins bind to sperm and not enough proteins are free to remove lipids from sperm surface. At higher concentrations, free BSPH1 can act as an acceptor to remove phospholipids and cholesterol from sperm membranes, which can lead to capacitation.

Based on these results, it is possible to assume that the proposed model for murine BSPH1’s role in capacitation would also apply to...
BSPH1 in human sperm capacitation (Plante et al., 2012). Briefly, in vivo, in the epididymis, BSPH1 is expressed in low concentration to allow binding of the protein on sperm surface without too much free protein to cause premature capacitation. BSPH1 coats the surface of the sperm and protects the membrane until sperm reach the oviduct where BSPH1 interacts with uterine/follicular fluid components such as HDL to remove the proteins, choline phospholipids such as PC and sphingomyelin, creating a phospholipid/cholesterol efflux and inducing capacitation. In vitro, the presence of increasing concentration of recombinant proteins would increase the level of free BSP proteins in the media and mimic the effect of the HDL, acting as acceptor to remove phospholipids and cholesterol from sperm membranes leading to capacitation. (Plante et al., 2012).

**Tyrosine phosphorylation**

In addition to changes in the membranes caused by the efflux of phospholipids and cholesterol, capacitation is often described as being associated with an increase in tyrosine phosphorylation (Naz et al., 1991). In human, two major proteins p105 (AKAP3) and p81 (AKAP4) are tyrosine phosphorylated during capacitation (Leclerc et al., 1997). The current study reveals that the addition of 30 μg/ml of rec-BSPH1 caused a significant decrease in the tyrosine phosphorylation level of p81, but the addition of 16 μg/ml of Trx-His-S caused a similar effect. This suggests that rec-BSPH1 is not responsible for the effect of tyrosine phosphorylation and that rec-BSPH1 had no impact on protein tyrosine phosphorylation. This is also supported by the absence of effect of rec-BSPH1 and Trx-His-S when sperm incubation was done in the presence of BSA (not shown). In the bovine, it was shown that tyrosine phosphorylation is increased in BSP-promoted capacitation in the presence of heparin but not in the presence of HDL (Lane et al., 1999). These results support the proposed mode of action for rec-BSPH1 in human sperm capacitation, which is more similar to the capacitation promoted by HDL.

**Hyperactivation and sperm motility**

In bovine sperm, BSP proteins can extend the motile life of sperm (Gwathmey et al., 2006). When compared with the control sample incubated in the absence of rec-BSPH1, sperm incubated with various concentrations of recombinant proteins showed no significant differences in total motility or progressive motility. Possibly, the interaction of BSPH1 with components in the female genital tract or with oviductal epithelium is necessary for the effect on sperm motility, as shown with bull sperm (Gwathmey et al., 2006).

Mammalian sperm commonly shows a distinctive motility pattern consisting of an increase in flagellar bend amplitude and beat asymmetry called hyperactivated motility (Suarez and Ho, 2003). Although there is no direct link, hyperactivation is often associated in a timely fashion with capacitation. Results with rec-BSPH1 show that even though sperm were capacitated after 4 h, no significant changes in the levels of hyperactivation were observed after 2 or 4 h incubation. It has been suggested that hyperactivation and capacitation are separate processes requiring similar conditions (Mortimer et al., 1998). It has also been suggested that hyperactivated motility is a capacitation-dependent event regulated by the signaling pathway involving tyrosine phosphorylation. Since rec-BSPH1 has no effect on tyrosine phosphorylation, it is likely that it has no effect on hyperactivation of sperm as well. It is, however, possible that the effect of rec-BSPH1 on hyperactivated motility is transient to be detected and that in vivo (in optimal conditions), BSPH1 could play a role not only in capacitation process but also in hyperactivation.

In conclusion, human rec-BSPH1 was expressed, purified and refolded. It bound to gelatin, heparin but not CSB and promoted sperm capacitation. Rec-BSPH1 had no impact on any motility parameters. This study is the first one to investigate the effect of human rec-BSPH1 on sperm function and to identify a possible biological role in human reproduction. Rec-BSPH1 bound to sperm over the equatorial segment and the post-acrosomal region both implicated in sperm–egg binding/fusion. The possible role of BSPH1 in these steps of fertilization remains to be studied. The results shown in this study for human rec-BSPH1 are similar to those previously observed with the murine rec-BSPH1 (Plante et al., 2012). These results confirm that the use of the mouse model to study the role of epididymal BSP proteins in sperm function can help to better understand the mechanisms implicating BSPH1 in human. The better understanding of the role played by BSPH1 in sperm functions could lead to the identification of a new factor with an impact on human male fertility and the development of new types of male contraceptives.

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**Authors’ roles**

G.P. contributed to the design, acquisition of data and analysis of the experiments related to Figs 1, 2, 4, 7 and 8. I.T. contributed to the design, acquisition of data and analysis of the experiments related to Figs 4, 5 and 7. C.L. contributed to the design, acquisition of data and analysis of the experiments related to Fig. 6. J.F. performed all bio-informatics analyses related to Fig. 3. P.L. contributed to the analysis and interpretation of the data of the experiments related to Fig. 6. P.M. contributed to the conception, design and analysis of all experiments. G.P. wrote the article. All authors revised the article and approved the final version for publishing.

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**Conflict of interest**

None declared.

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