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Rôle de protéines épididymaires humaines et murines dans les fonctions spermatiques

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Résumé

L'infertilité affecte jusqu'à 15-20% des couples en âge de se reproduire. C'est pourquoi, mieux comprendre les mécanismes à la base de la fécondation est essentiel pour l'identification de nouvelles causes d'infertilité et l'optimisation des techniques de reproduction assistée. La capacitation est une étape de la maturation des spermatozoïdes qui se déroule dans le tractus génital femelle. Elle est requise pour la fécondation d'un ovocyte. Notre laboratoire a démontré que des protéines du plasma séminal bovin, appelées protéines Binder of SPerm (BSP), se lient aux phospholipides portant des groupements choline à la surface de la membrane des spermatozoïdes lors de l'éjaculation et promeuvent la capacitation. Ces protéines exprimées par les vésicules séminales sont ubiquitaires chez les mammifères et ont été étudiées chez plusieurs espèces dont l'étalon, le porc, le bouc et le bélier. Récemment, l'expression de gènes homologues aux BSP a été découverte dans les épididymes d'humains (*BSPH1*) et de souris (*Bsph1* et *Bsph2*). Notre hypothèse est que les BSP chez ces deux espèces sont ajoutées aux spermatozoïdes lors de la maturation épididymaire et ont des rôles dans les fonctions spermatiques, similaires à ceux des protéines BSP bovines.

Les protéines BSP humaines et murines représentent une faible fraction des protéines totales du plasma séminal. Pour cette raison, afin d'étudier leurs caractéristiques biochimiques et fonctionnelles, des protéines recombinantes ont été produites. Les protéines recombinantes ont été exprimées dans des cellules *Escherichia coli* origami B(DE3)pLysS en utilisant un vecteur d'expression pET32a. Suivant la lyse cellulaire, les protéines ont été dénaturées avec de l'urée et purifiées par chromatographie d'affinité sur ions métalliques immobilisés. Une fois liées à la colonne, les protéines ont été repliées à l'aide d'un gradient d'urée décroissant avant d'être éluées. Cette méthode a mené à la production de trois protéines recombinantes (rec-BSPH1 humaine, rec-BSPH1 murine et rec-BSPH2 murine) pures et fonctionnelles.

Des expériences de chromatographie d'affinité et de co-sédimentation nous ont permis de démontrer que les trois protéines peuvent se lier à des ligands connus des protéines BSP comme la gélatine et l'héparine en plus de pouvoir se lier aux spermatozoïdes. Nos études ont également révélées que les deux protéines rec-BSPH1 peuvent se lier aux liposomes de

phosphatidylcholine (PC) et sont capable de promouvoir la capacitation des spermatozoïdes. À l'opposé, rec-BSPH2 ne peut ni se lier aux liposomes de PC, ni stimuler la capacitation. Finalement, les protéines recombinantes n'ont aucun effet sur la réaction acrosomique ou sur la motilité des spermatozoïdes.

Chez les bovins, les protéines BSP induisent la capacitation grâce des interactions avec les lipoprotéines de haute densité (HDL) et les glycosaminoglycanes. Puisque le HDL est également un joueur important de la capacitation chez la souris, le rôle de la protéine native BSPH1 murine au niveau de la capacitation induite par le HDL a été étudié. Les résultats obtenus suggèrent que, *in vivo*, la protéine BSPH1 de souris serait impliquée dans la capacitation via une interaction directe avec le HDL. Comme les protéines BSPH1 humaines et murines sont orthologues, ces résultats pourraient aussi s'appliquer à la fertilité humaine.

Les résultats présentés dans cette thèse pourraient mener à une meilleure compréhension de la fertilité masculine et aider à améliorer les techniques de reproduction assistée. Ils pourraient également mener au développement de nouveaux tests diagnostiques ou de contraceptifs masculins.

Mots-clés : Infertilité masculine, Épididyme, Protéines Binder of SPerm, Capacitation, Protéines recombinantes, Lipoprotéines de haute densité.

Abstract

Infertility can affect as much as 15-20% of couples of reproductive age. Therefore, elucidating mechanisms occurring during fertilization is needed to resolve cases of infertility and optimize assisted reproductive technology procedures. Sperm capacitation is a maturation step that takes place in the female genital tract and is deemed to be essential for sperm to fertilize an oocyte. Our laboratory has demonstrated that proteins from bovine seminal plasma called Binder of SPerm (BSP) proteins bind to choline phospholipids on the sperm membrane upon ejaculation and promote capacitation. These proteins expressed in seminal vesicles are ubiquitous amongst mammals and have been studied in many species including stallion, boar, ram and goat. More recently, the expression of BSP-homologous genes has been discovered in the epididymis of humans (*BSPH1)* and in mice (*Bsph1* and B*sph2)*. We hypothesized that the BSP homologs in these two species are added to sperm during epididymal maturation and play similar roles in sperm functions as bovine BSP proteins.

BSP proteins in humans and mice constitute only a minute percentage of the seminal plasma proteins. Thus, to study their biochemical and functional characteristics recombinant proteins were produced. Recombinant proteins were expressed in E*scherichia coli* origami B(DE3)pLysS cells using a pET32a expression vector. Following cell lysis, proteins were denatured using urea and purified by immobilized metal ion affinity chromatography. Once bound to the resin, proteins were refolded using a decreasing urea gradient after which they were eluted. This method led to the production of three pure, functional recombinant proteins (human rec-BSPH1, mouse rec-BSPH1 and mouse rec-BSPH2).

Using affinity chromatography and co-sedimentation experiments, we were able to demonstrate that all three recombinant proteins bind known ligands of BSP proteins including gelatin, heparin and have the ability to bind to sperm. Studies also revealed that both rec-BSPH1 proteins bind to phosphatidylcholine (PC) liposomes and promote sperm capacitation. However, rec-BSPH2 neither binds to PC liposomes nor stimulates capacitation. Recombinant proteins had no effect on acrosome reaction or sperm motility.

In bovine, BSP proteins promote sperm capacitation through interactions with high-density lipoproteins (HDL) and glycosaminoglycans. Since in mice HDL is also a major factor

implicated in capacitation, the role of the native murine BSPH1 protein in HDL-induced capacitation was investigated. Results obtained suggest that, *in vivo*, murine BSPH1 protein could act in capacitation via a direct interaction with HDL. As human and murine BSPH1 are orthologs, these results could possibly also apply to human fertility.

The results presented in this thesis could lead to a better understanding of male fertility and help improve assisted reproduction technology procedures. They could also lead to the development of diagnostic tests as well male contraceptives.

Keywords: Male fertility, Epididymis, Binder of SPerm (BSP) proteins, Capacitation, Recombinant proteins, High-density lipoproteins.

Table des matières

Liste des figures

Liste des sigles et abréviations

~	Approximativement
%	Pourcent
µl	Microlitre
µg	Microgramme
µM	Micromolaire
°C	Degré Celcius
ABC	ATP-Binding Casette
ADAM	« A disintegrin And metalloprotease »
ADN	Acide Désoxyribonucléique
Akt	Protéine Kinase B
AMPc	Adénosine monophosphate cyclique
AR	Réaction acrosomique
BSA	Albumine de sérum bovin (Bovine serum albumin)
BSP	Binder of SPerm
BSPH	Binder of SPerm Homolog
BWW	Milieu Biggers-Whitten-Whittingham
Ca^{2+}	Ion calcique
CD-36	Cluster determinant 36
CHO	Cholestérol
cm	Centimètre
CRISP	Cysteine-rich secretory proteins
CSB	Chondroïtine sulfate B
ELISA	Enzyme-linked immunosorbant assay
E-RABP	Epididymal retinoic acid binding protein
ERK	Kinases régulées par des signaux extracellulaires
FITC	Isothiocyanate de fluorescéine (Fluorescein isothiocyanate)
FIV	Fécondation *in vitro*
Fn2	Domaine de type II de la fibronectine
FSH	Hormone folliculo-stimulante

GAG	Glycosaminoglycane
GPI	Glycosylphosphatidylinositol
h	Heure
HCO_3^-	Bicarbonate
HDL	Lipoprotéines de haute densité (High-density lipoproteins)
His_6	Hexahistidine
IA	Insémination artificielle
IgG	Immunoglobuline G
IICS	Injection intra-cytoplasmique de spermatozoïde
IMAC	Chromatographie d'affinité sur ions métalliques immobilisés (Immobilized metal ion affinity chromatography)
K^+	Ion potassium
kcal	Kilocalorie
kDa	Kilodalton
KO	Knock-out
LDL	Lipoprotéines de faible densité
LH	Hormone lutéinisante
M	Molaire
mg	Milligramme
min	Minute
ml	Millilitre
mM	Millimolaire
Na^+	Ion sodique
Ni^{2+}	Ion de nickel
NRS	Sérum pré-immun de lapin (Pre-immune rabbit serum)
OMS	Organisation mondiale de la santé
PC	Phosphatidylcholine
PCR	Amplification en chaîne par polymérase (Polymerase chain reaction)
PE	Phosphatidylethanolamine
PEBP1	Phosphatidylethanolamine binding protein 1

pH	Potentiel d'hydrogène
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3-kinase
PKA	Protéine kinase A
PKC	Protéine kinase C
PLA_2	Phospholipase A2
PSA	Agglutinine de Pisum sativum (Pisum sativum agglutinin)
PVDF	Polyfluorure de vinylidène (Polyvinylidene fluoride)
Rec-BSPH	Protéine recombinante Binder of SPerm Homolog
ROS	Espèces réactives de l'oxygène
rpm	Rotation par minute
s	Seconde
sAC	Adenylyl cyclase soluble
SM	Sphingomyéline
SR-BI	Scavenger receptor class B type I
TCA	Acide trichloroacétique (Trichloric acid)
TRA	Techniques de reproduction assistée
TRITC	Isothiocyanate de tramethylrhodamine
Trx	Thioredoxine
WH	Whittens-HEPES media
ZP	Zone pellucide

Venerunt, vidi, intelleximus

«Nothing is impossible, the word itself says I'm possible » - Audrey Hepburn

« L'art de la réussite consiste à savoir s'entourer des meilleurs » - John F. Kennedy

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Introduction

1. Mise en contexte

La reproduction est la base de la vie et une nécessité pour la survie des espèces. Au cours des dernières décennies, un déclin alarmant de la fertilité humaine a été observé. En effet, les dernières statistiques montrent qu'au Canada, 16% des couples sont touchés par l'infertilité, ce qui représente environ un couple sur six [1]. Ce nombre représente le double de ce qui était observé au cours des années 1980 [1]. L'infertilité est considérée comme une maladie par l'Organisation mondiale de la Santé (OMS). Elle est définie comme étant l'incapacité d'un couple à procréer ou à mener une grossesse à terme au bout d'une année ou plus de rapports sexuels réguliers non protégés [2]. Bien qu'elle ne soit pas une affection fatale pour les individus touchés, l'infertilité peut avoir sur eux un impact psychologique important, entraînant des périodes de stress intenses, des troubles anxieux et même des dépressions [3].

On estime que des facteurs masculins sont en cause dans 40 à 50% des cas d'infertilité. Au niveau mondial, cela équivaut à environ un homme sur vingt souffrant de problèmes de fertilité [1, 4]. Les sources de l'infertilité masculine sont très variées. Elle peut être causée entre autre par des affections congénitales, des infections (ex : virus du papillome humain), une exposition à des agents chimiques (ex : agents chimio-thérapeutiques, phtalates ou bisphénol A) ou des mauvaises habitudes de vie (ex : tabac, abus d'alcool ou obésité) [5-8]. L'infertilité masculine résulte souvent en un nombre réduit de spermatozoïdes, une motilité réduite ou une morphologie anormale des spermatozoïdes, ou encore en une incapacité pour un spermatozoïde de reconnaitre et d'interagir avec l'ovocyte [9]. Toutefois, 30 à 40% des hommes se présentant dans une clinique de fertilité recevront un diagnostic d'infertilité idiopathique (i.e. de cause inconnue) [10, 11]. Il est donc important de mieux comprendre les mécanismes moléculaires à la base de la reproduction afin de permettre le développement de traitements appropriés pour ces hommes.

Depuis plusieurs années, les couples souffrant d'infertilité peuvent avoir recours aux techniques de reproduction assistée (TRA) pour concevoir un enfant. Les méthodes utilisées pour contourner le ou les problèmes de fertilité incluent l'insémination artificielle (IA), la fécondation *in vitro* (FIV) et l'injection intra-cytoplasmique de spermatozoïde (IICS) [12 , 13].

L'IA est la méthode la plus simple et la moins invasive. Cette méthode consiste à injecter artificiellement du sperme frais, traité ou congelé dans la cavité utérine de la femme au moment de l'ovulation (naturelle ou provoquée). Le FIV est la méthode la plus utilisée en clinique de fertilité. Pour cette méthode, les ovocytes de la femme sont prélevés et mis en contact avec des spermatozoïdes en laboratoire. Les ovocytes fécondés sont ensuite mis en culture *in vitro* pour quelques jours avant d'être transférés à nouveau dans la cavité utérine de la femme ou d'une mère porteuse. Dans les cas où les spermatozoïdes sont incapables de féconder spontanément un ovocyte *in vitro* ou que le nombre de spermatozoïdes disponibles est très faible, il est possible d'utiliser l'IICS. Cette méthode est très semblable à la FIV, mais plutôt que d'incuber les ovocytes en présence de spermatozoïdes, un spermatozoïde unique est prélevé puis injecté directement dans un ovocyte.

Depuis le premier succès de FIV en 1978, le nombre de naissances annuelles par TRA est en croissance constante. On estime maintenant que chaque année, 2 à 3% des naissances sont le résultat de TRA et que le nombre d'enfants conçu par TRA à travers le monde a dépassé les 5 millions en 2012 [14]. Malgré ce nombre très élevé, on ignore toujours l'ampleur des effets possiblement nocifs à court et à long terme des procédures de TRA sur les mères et leur progéniture. Toutefois, les études réalisées sur les effets des TRA semblent démontrées qu'elles ne sont pas inoffensives. La FIV et l'IICS sont des méthodes très invasives qui contournent plusieurs étapes de sélection naturelle présentes lors des processus naturel de fécondation [15]. Des études ont démontré que, lors de la FIV et l'IICS, le transfert des embryons est lié à 50-70% de risques additionnels de naissances prématurées et de malformations congénitales. Les enfants résultants de FIV présentent également plus de risques de développer une pression artérielle élevée, des problèmes vasculaires ou encore des taux de glucose sanguin élevés [14, 16, 17]. Finalement, on rapporte, chez les enfants conçus par IICS, une incidence d'anomalies chromosomiques plus élevée que chez les enfants conçus naturellement [18].

Même si certains programmes permettent maintenant le remboursement des frais associés aux traitements de reproduction assistée pour certains couples, il n'en demeure pas moins que les coûts des TRA sont très élevés et peuvent atteindre jusqu'à 30% des revenus annuels d'un couple au Canada. En général, un cycle de FIV coûte approximativement 8,500$ [19, 20]. En dépit de ces coûts élevés, seulement 30% des cycles de FIV produiront un

enfant viable [20]. C'est pourquoi, il est important d'approfondir la compréhension des mécanismes à la base de la reproduction ce qui devrait permettre de développer de nouveaux tests diagnostiques, réduire le nombre de cas d'infertilité idiopathique, développer des nouvelles TRA, améliorer les méthodes existantes et réduire les coûts associés à ces traitements.

Dans un autre ordre d'idées, les sociétés modernes sont également aux prises avec un nombre impressionnant de grossesses non désirées. De nombreuses méthodes de contraception existent sur le marché, que ce soit des méthodes mécaniques (condoms, stérilets et diaphragmes), chimiques (spermicides) ou hormonales (comprimés et injections). Toutefois, en excluant le condom, on remarque un grand manque de contraceptifs masculins. Malgré toutes les méthodes de contraception disponibles, on estime qu'un tiers des grossesses annuelles dans le monde ne sont pas planifiées et que plus de 21% de ces grossesses non planifiées, soit environ 44 millions, sont avortées [21]. Même dans les pays industrialisés, la moitié de ces avortements se font dans des conditions non sécuritaires qui mettent la vie des mères en danger [21]. Des études récentes démontrent que des moyens de contraceptions plus efficaces permettraient d'éviter annuellement plus de 50 millions de grossesses non planifiées, 22 millions d'avortements et 79 000 morts maternelles liées à l'avortement [22]. Une meilleure compréhension des mécanismes à la base de la reproduction pourrait donc également permettre de développer des nouvelles méthodes efficaces de contraception.

Pour toutes ces différentes raisons, cette thèse portera sur la fertilité masculine chez l'humain et la souris et sur les mécanismes qui, suivant la synthèse des spermatozoïdes, permettent leur maturation et les rendent aptes à féconder un ovocyte.

2. Les gamètes

Chez les mammifères, la reproduction se fait de manière sexuée. De ce fait, elle implique la participation de deux individus parentaux de même espèce et de sexes opposés. Chaque individu parental fournit un gamète haploïde qui contient une seule copie du génome de l'espèce. Au moment de la fécondation, le gamète mâle (le spermatozoïde) fusionne avec le gamète femelle (l'ovocyte) afin de donner un zygote diploïde (possédant deux copies du génome). Suite à de multiples divisions, ce zygote deviendra un individu génétiquement différent des deux parents.

2.1 L'ovocyte

L'ovogénèse est le processus de production d'ovocytes qui se déroule dans les ovaires (Figure 1). Elle débute lors du développement embryonnaire lorsque les cellules souches germinales se trouvant dans les ovaires entament les premières étapes de la méiose. La méiose, le procédé permettant la division d'une cellule diploïde en deux cellules haploïdes, n'est toutefois pas complétée au niveau embryonnaire. Elle est plutôt bloquée jusqu'à la puberté. Jusqu'à la fin de leur maturation, les ovocytes immatures sont conservés à l'intérieur de petites cavités ovariennes appelées follicules. Suivant le développement embryonnaire, les ovaires contiennent des millions de follicules et autant d'ovocytes immatures. Cependant, seulement quelques centaines de ces ovocytes deviendront complètement matures et auront la capacité d'être fécondés [23, 24].

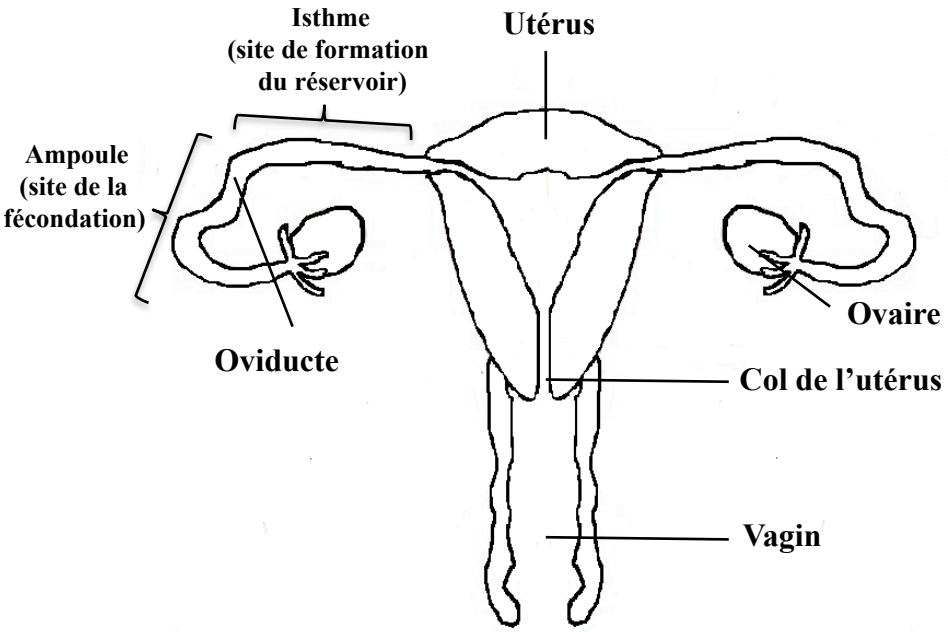

Figure 1 : Représentation schématique du système reproducteur femelle chez l'humain. (Adapté de [25])

Les ovocytes ne deviennent pas tous matures en même temps. Suivant la puberté, leur maturation s'effectue plutôt de manière cyclique en réponse à des variations hormonales. Ces cycles se poursuivent jusqu'à la ménopause. Un cycle ovarien dure entre 4 et 5 jours chez la souris et environ 28 jours chez l'humain [26]. Il débute lorsqu'un ou plusieurs ovocytes

immatures, selon l'espèce, entament les dernières étapes de la division méiotique et se termine lors de l'expulsion des ovocytes matures. Il y a alors rupture des follicules ovariens ce qui permet le relâchement du liquide folliculaire et des ovocytes matures dans les trompes utérines (oviducte).

Un ovocyte fait environ 300 fois la taille d'un spermatozoïde. Il possède un gros cytosol contenant des mitochondries, des ribosomes, des molécules du cytosquelette (actine et microtubules) et un noyau haploïde. Le tout est entouré d'une membrane plasmique. L'ovocyte mature est également entouré de deux structures : le cumulus oophorus et la zone pellucide (ZP) (Figure 2) [23].

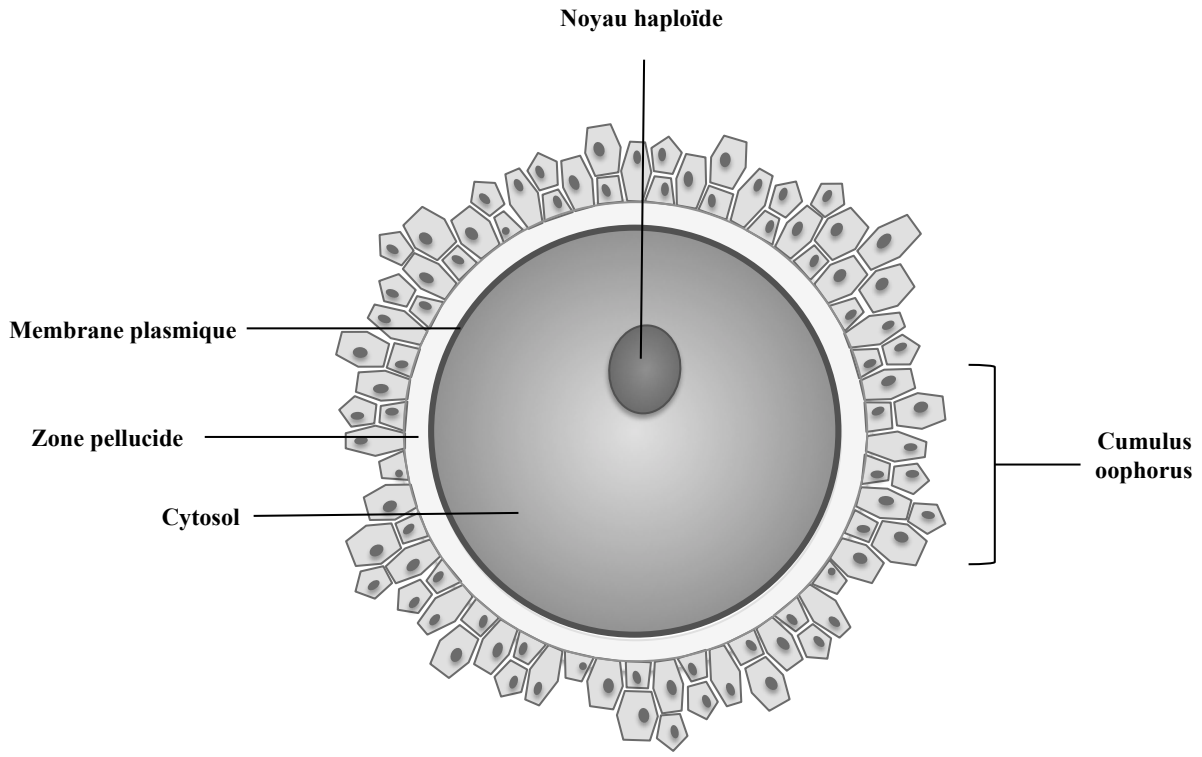

Figure 2: Représentation schématique d'un ovocyte.

Le cumulus oophorus est la première couche rencontrée par le spermatozoïde lors de la fécondation. Il est composé des cellules du cumulus et d'une matrice constituée principalement d'acide hyaluronique. La ZP qui se trouve entre le cumulus oophorus et la membrane plasmique est quant à elle composée de glycoprotéines. On retrouve quatre de ces glycoprotéines chez l'humain (ZP1, ZP2, ZP3 et ZP4) alors que la souris ne possède que les trois premières. Les protéines ZP2, ZP3 et ZP4 sont toutes trois responsables de la

reconnaissance du spermatozoïde. ZP3 est toutefois le récepteur primaire responsable de l'interaction avec le spermatozoïde et de l'induction de la réaction acrosomique. ZP2 serait quant à elle importante pour empêcher la polyspermie, c'est-à-dire la fécondation d'un ovocyte par plus d'un spermatozoïde [27, 28]. De son côté, la protéine ZP1 sert de matrice liant les protéines ZP les unes aux autres [29].

2.2 Le spermatozoïde

Les spermatozoïdes sont des cellules hautement spécialisées qui doivent traverser de longues distances, et subir de multiples modifications lors de leur parcours dans les voies génitales mâle (Figure 3) et femelle (Figure 1) avant de pouvoir jouer leur rôle dans la fécondation. Ils sont premièrement formés dans les testicules et deviennent matures lors de leur long transit dans l'épididyme (section 3) où ils sont entreposés jusqu'à l'éjaculation. Au moment de l'éjaculation, les spermatozoïdes subissent de nouvelles modifications lorsqu'ils entrent en contact avec les sécrétions des glandes accessoires (section 4). Finalement, une fois dans le tractus génital femelle, les spermatozoïdes obtiennent leur pouvoir fécondant dans un processus appelé la capacitation (section 5) ce qui leur permet de subir la réaction acrosomique et de féconder un ovocyte (section 6).

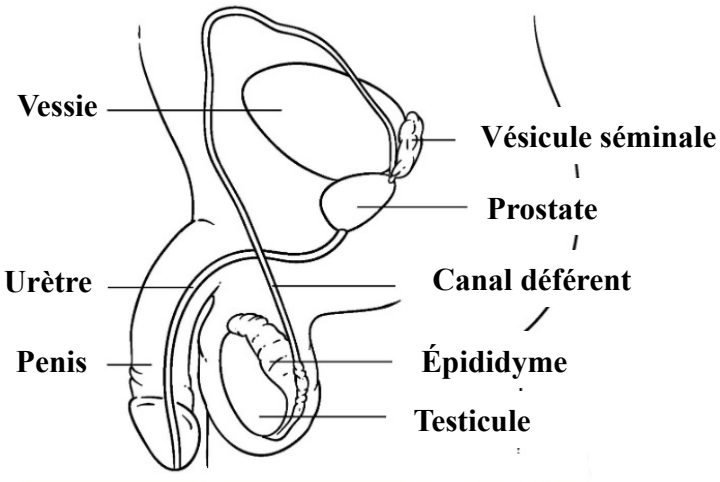

Figure 3 : Représentation schématique du système reproducteur mâle chez l'humain. (Adapté de [30])

2.2.1 Synthèse

La synthèse des spermatozoïdes, se fait dans les testicules, au niveau des tubules séminifères et dépend de deux types cellulaires retrouvés dans le testicule; les cellules de Sertoli et les cellules de Leydig. Les cellules de Sertoli tapissent l'intérieur des tubules séminifères formant une couche unilaminaire et fournissent des nutriments et un support structural aux cellules en synthèse. Les cellules de Leydig sont, quant à elles, responsables de la production d'hormones stéroïdiennes nécessaires pour la différentiation cellulaire [31]. Elles produisent jusqu'à 95% de la testostérone produite chez l'homme. Contrairement à l'ovogénèse qui est cyclique, la synthèse des spermatozoïdes est un processus continu qui se poursuit jusqu'à la mort de l'individu [32].

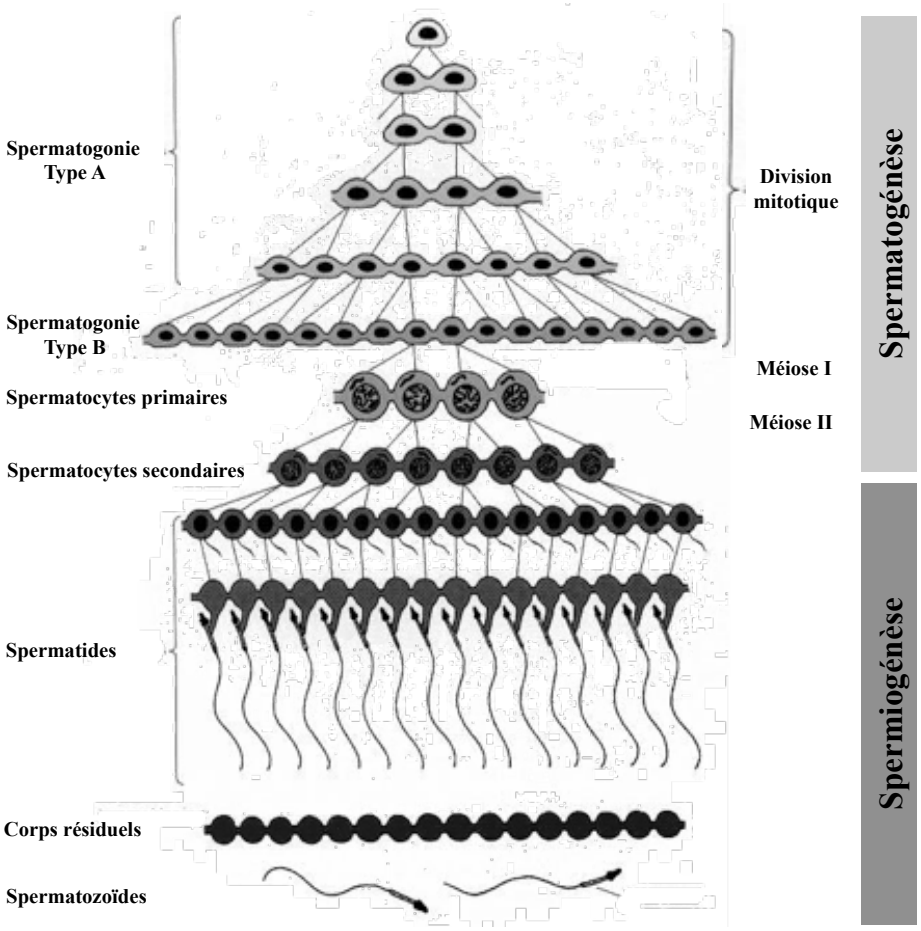

Figure 4: Représentation schématique de la synthèse des spermatozoïdes

(Adapté de [33])

La synthèse de spermatozoïdes se fait en deux grandes étapes soit la spermatogénèse et la spermiogénèse (Figure 4). La spermatogénèse est le processus durant les cellules souches germinales, appelées les spermatogonies, subissent une série de divisions mitotiques, se différencient, puis entre en méiose pour finalement devenir des cellules haploïdes appelées spermatides [33]. Les spermatogonies font leur apparition dans le testicules durant le développement embryonnaire, mais n'entrent pas en méiose avant la puberté lorsque la production de certaines hormones augmente. En effet, la régulation de la spermatogénèse fait intervenir plusieurs hormones. Par exemple, l'hormone lutéinisante (LH) et l'hormone folliculo-stimulante (FSH) sécrétées par l'hypophyse agissent directement sur les cellules de Leydig et les cellules de Sertoli respectivement, ce qui engendre la production de testostérone par les cellules de Leydig et la sécrétion d'androgen binding protein par les cellules de Sertoli augmentant la concentration de testostérone testiculaire et la différenciation cellulaire [34].

Une fois la méiose complétée, la spermiogénèse, l'étape de différenciation des spermatides en spermatozoïdes, débute. Les cellules se transforment alors et adoptent une structure complètement différente. Le flagelle et l'acrosome du spermatozoïde se forment, l'ADN se condense, les histones sont remplacées par des protamines, le cytoplasme est réduit et la plupart des organelles incluant le Golgi, le réticulum endoplasmique, les lysosomes et les peroxisomes sont perdues [35]. Toutes ces modifications ont pour but de réduire la consommation inutile d'énergie par le spermatozoïde qui doit conserver toute son énergie pour atteindre et féconder un ovocyte. En raison de ces changements, les spermatozoïdes sont incapables de synthétiser des protéines ou de recycler les protéines présentes à leur surface. Ils sont transcriptionnellement inactifs [35]. Les spermatozoïdes diffèrent aussi des autres types cellulaires de par leur capacité à se mouvoir et à résister à plusieurs changements de conditions drastiques lors de leur transit à travers les tractus génitaux du mâle et de la femelle. Ces changements de conditions incluent des changements d'osmolarité, de pH et de température [36, 37].

Les scientifiques ont longtemps pensé que la fonction unique du spermatozoïde était de fournir la moitié du génome d'un embryon. Toutefois, des recherches récentes démontrent que qu'il pourrait également jouer un rôle important au niveau de l'organisation et du développement préimplantatoire de l'embryon, suite à la fécondation [38]. En effet, en plus de fournir une partie du bagage génétique à l'embryon, il a été montré que le spermatozoïde

fournissait également des ARN messagers, importants pour le développement embryonnaire, et un centriole. Selon certaines études, le centriole serait important pour la segmentation embryonnaire; la première division mitotique de l'embryon. Il a été démontré qu'un mauvais transfert du centriole pouvait mener à une segmentation trop précoce ce qui a des conséquences néfastes pour les embryons et mène souvent à un arrêt de la division embryonnaire [39].

Plusieurs variations existent entre les spermatozoïdes des différentes espèces. Des différences ont été rapportées au niveau de la morphologie du spermatozoïde, des protéines de surface, de la composition des membranes plasmiques, du nombre de spermatozoïdes produits, de la composition du plasma séminal et du volume des éjaculats [40]. Ils ont néanmoins tous une structure similaire qui se divise en deux grandes parties soit le flagelle et la tête.

2.2.2 Structure

Le flagelle est la partie permettant aux spermatozoïdes de se déplacer. Il est divisé en quatre parties soit le col, la pièce intermédiaire, la pièce principale et la pièce terminale (Figure 5A). La structure principale du flagelle se nomme l'axonème. Il s'agit d'une structure circulaire hautement conservée chez les mammifères, composée de 9 paires de microtubules entourant deux microtubules centraux. C'est le glissement des microtubules les uns sur les autres qui engendre les battements du flagelle permettant aux spermatozoïdes de se mouvoir. Des structures secondaires entourent l'axonème et permettent de définir les différents segments du flagelle. Ces structures incluent deux centrioles au niveau du col, des mitochondries au niveau de la pièce intermédiaire, des fibres denses externes au niveau des pièces intermédiaire et principale, une gaine fibreuse au niveau de la pièce principale et une membrane plasmique qui couvre le flagelle en entier [41].

Afin de se déplacer, les spermatozoïdes ont besoin de beaucoup d'énergie. Une partie de cette énergie est produite par les mitochondries, par phosphorylation oxydative [42]. Le reste de l'énergie provient de la glycolyse qui se fait au niveau de la pièce principale ; le segment du flagelle le plus riche en enzymes glycolytiques [42]. Les fibres denses externes et la gaine fibreuse ont plutôt des rôles structuraux. Elles servent entre autre à la protection de l'axonème et au maintien de l'élasticité du flagelle. La gaine fibreuse sert également à l'échafaudage d'enzymes glycolytiques et de protéines de signalisation [41].

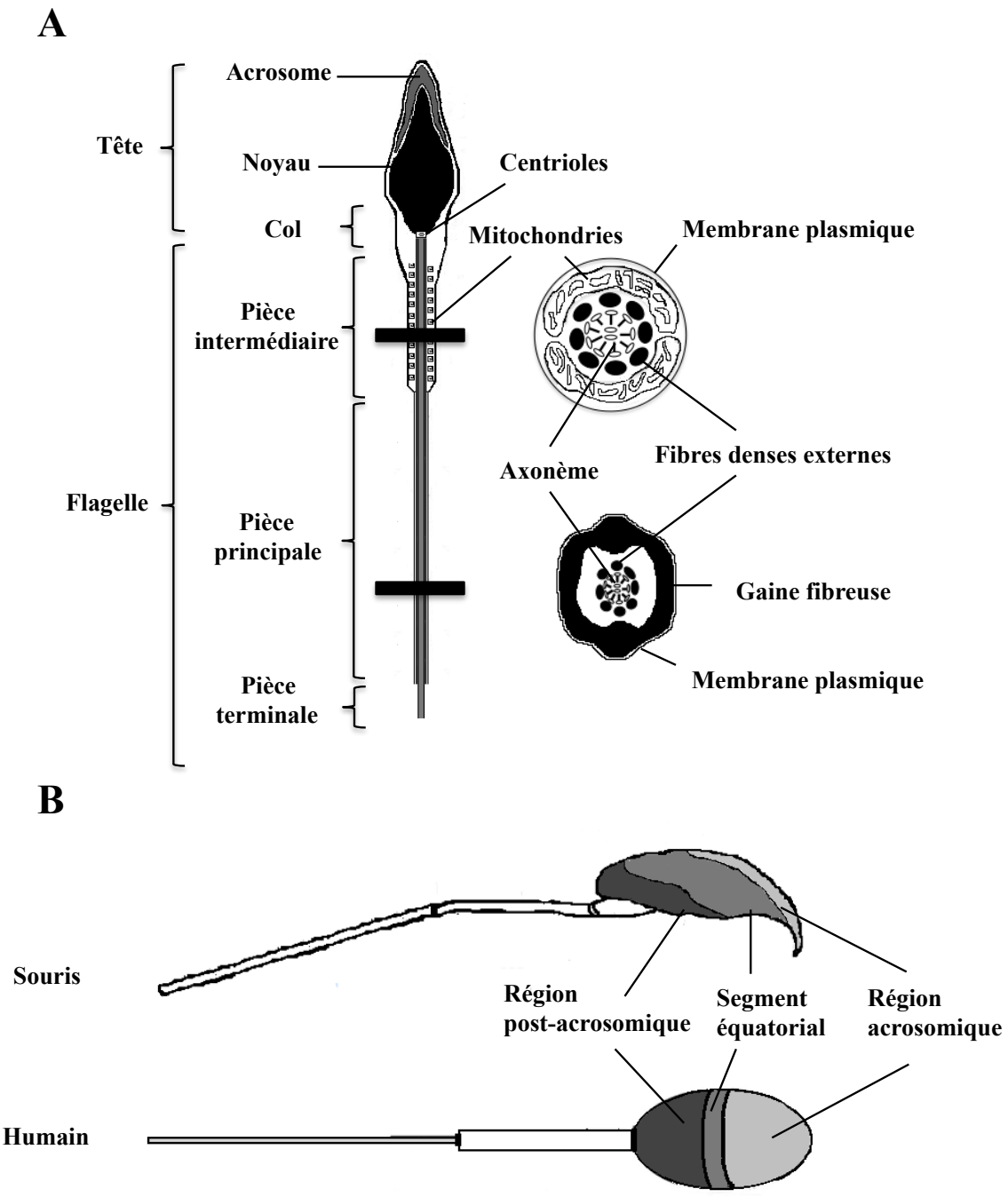

Figure 5 : Représentation schématique du spermatozoïde (A) Structures principales du spermatozoïde humain vu de côté et coupe transversale du flagelle. (B) Régions de la membrane plasmique des spermatozoïdes humains et de souris vu de face. (Adapté de [41, 43, 44])

La tête du spermatozoïde est la partie contenant le bagage génétique. Elle contient le noyau d'ADN et un très petit cytosol. À la partie la plus apicale du spermatozoïde se trouve l'acrosome qui contient des enzymes (hyaluronidase, neuraminidase et proacrosine) nécessaires au passage du spermatozoïde à travers la ZP lors de la fécondation. Le tout est entouré d'une membrane plasmique (Figure 5A). Au niveau de la membrane plasmique, la tête se divise en trois grandes régions : la région acrosomique, le segment équatorial et la région post-acrosomique. La région acrosomique recouvre l'acrosome, alors que les régions équatoriale et post-acrosomale sont les lieux de contact initiaux entre le spermatozoïde et l'ovocyte et contiennent entre autres des protéines nécessaires pour la fusion des gamètes (Figure 5B) [23].

2.2.3 Composition lipidique

La membrane plasmique des spermatozoïdes est très différente de celle des autres types cellulaires. Dans la plupart des cellules de mammifères, les lipides peuvent diffuser librement dans les membranes. Dans les spermatozoïdes sortant des testicules, une grande partie des lipides n'a plus la capacité de diffuser. Cette fraction augmente lors du passage dans l'épididyme dépassant jusqu'à 50% des lipides qui ne diffuse plus [23]. Le contenu lipidique de la membrane plasmique des spermatozoïdes est également différent des autres types cellulaires étant plus riche en plasmalogène (20-40%) et possédant une forte proportion de phospholipides (~70%) [23].

En général, les phospholipides les plus abondants de la membrane plasmique des spermatozoïdes sont la phosphatidylcholine (PC), la phosphatidylethanolamine (PE) et la sphingomyéline (SM). Toutefois, les proportions de ces lipides varient d'une espèce à l'autre. Chez le taureau, la souris et le porc, le phospholipide le plus abondant est la PC alors que chez l'humain la PE est plus présente que la PC [23, 45-47]. Les ratios cholestérol/phospholipides varient également entre les espèces allant de 0.25 chez la souris à plus de 0.83 chez l'humain [45, 48]. Chez toutes les espèces, la maturation épididymaire et la capacitation modifient la concentration et la distribution des lipides du spermatozoïde causant une diminution du ratio cholestérol/phospholipides et des concentrations de phospholipides [23].

La membrane plasmique des spermatozoïdes contient également des microdomaines riches en cholestérol, en glycosphingolipides et en protéines à ancrage

Glycosylphosphatidylinositol (GPI) qui sont résistants à de faibles concentrations de détergents non-ioniques. Ces domaines sont appelés radeaux lipidiques ou « lipid rafts » [23]. Les protéines qu'ils contiennent permettent entre autres la régulation de la signalisation intracellulaire et des fonctions cellulaires. Les expériences réalisées au cours des dernières années suggèrent que les radeaux lipidiques seraient importants pour la régulation de la capacitation, la réaction de l'acrosome et l'interaction du spermatozoïde avec l'ovocyte [49].

2.2.4 Composition protéique

Les spermatozoïdes contiennent plusieurs milliers de protéines incluant des protéines de structures et des protéines nucléaires mais aussi des protéines servant à la motilité des spermatozoïdes, à la production d'énergie, à la signalisation cellulaire, à la capacitation, à la réaction de l'acrosome et à l'interaction du spermatozoïde avec l'ovocyte [50]. Bien que les spermatozoïdes possèdent beaucoup de protéines à leur sortie des testicules, la majorité des protéines sont acquises durant la maturation épididymaire et lors de l'éjaculation. Chez l'humain, on estime que le protéome du spermatozoïde contient 7500 protéines. Six mille cent quatre-vingt-dix-huit de ces protéines ont déjà été identifiée et uniquement 30% d'entre elles ont une origine testiculaire [51]. Chez la souris moins de protéines ont été rapportées. L'étude du protéome de spermatozoïdes épididymaires murins la plus récente rapportait l'identification d'environ 2850 protéines [52]. L'étude des protéines associées aux spermatozoïdes est importante afin de mieux comprendre les étapes clés de la maturation des spermatozoïdes, d'identifier des cibles pour la contraception et d'identifier des causes d'infertilité [53].

3. L'épididyme

L'épididyme est un organe se situant au dessus du testicule. Il s'agit d'un long tube enroulé sur lui-même mesurant jusqu'à un mètre chez la souris et six mètres chez l'humain [54]. L'épididyme se divise en quatre grandes parties : le segment initial, la tête (caput), le corps (corpus) et la queue (cauda) (Figure 6) Toutefois, ces quatre parties peuvent également être divisées en plusieurs segments, une division qui diffère d'une espèce à l'autre. Par exemple, les épididymes de souris et de rats sont divisés en un total de 10 et 19 segments respectivement [55]. Chacun de ces segments diffère du précédent par sa concentration en

ions, son pH, l'osmolalité du fluide épididymaire qu'il contient et par les différents gènes qui y sont exprimés.

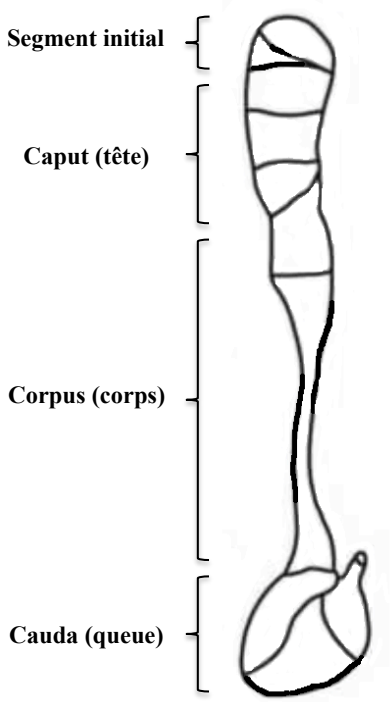

Figure 6 : Représentation schématique de l'épididyme de souris (Adapté de [55])

3.1 Maturation épididymaire

Le transit des spermatozoïdes dans l'épididyme dure environ 10 jours peu importe l'espèce. Les spermatozoïdes sortent du testicule par le canal afférent et se retrouvent dans le segment initial. Dans la tête de l'épididyme, une forte réabsorption d'eau permet de concentrer les spermatozoïdes qui sont ensuite transportés de manière passive, c'est-à-dire sans dépense d'énergie, tout au long de l'épididyme [56]. C'est lors de leur passage dans l'épididyme que les spermatozoïdes acquièrent leur motilité, deviennent aptes à subir la capacitation et acquièrent l'habileté à reconnaître et féconder un ovocyte [57]. Ils subissent également des modifications membranaires et acquièrent plusieurs protéines incluant des facteurs décapacitants. Ces facteurs se lient à la surface des spermatozoïdes et permettent de prévenir une capacitation et une réaction acrosomique précoce.

Le long trajet au travers de l'épididyme se termine dans la partie cauda où les spermatozoïdes sont entreposés jusqu'à l'éjaculation [56]. Dans la queue de l'épididyme, les spermatozoïdes sont conservés dans un état quiescent et sont incapables de se mouvoir, toujours dans le but d'éviter les dépenses d'énergie inutiles. Cet état quiescent est provoqué par la diminution du pH, l'augmentation de l'osmolalité et la diminution de la concentration des ions Na^+, K^+, Ca^{2+} et HCO_3^- dans le fluide épididymaire de ce segment [58].

3.2 Protéines majeures de l'épididyme

Les protéines exprimées dans les épididymes sont cruciales pour la fertilité et jouent des rôles très variés. Ces rôles incluent entre autres la réabsorption d'eau, les modifications membranaires, la production d'énergie, la motilité du spermatozoïde, la capacitation et l'interaction spermatozoïde-ovocyte [52, 59]. La majorité des protéines épididymaires, entre 60 et 83% selon l'espèce, sont sécrétées dans la région proximale de l'épididyme, au niveau de la tête [56]. On retrouve parmi les protéines majeures de cet organe des lipocalines, des protéines liant les lipides, des glycosidases, des enzymes antioxydants, des protéases et des clusterines [56].

Les lipocalines sont des protéines se liant à des petites molécules lipophiles. La protéine E-RABP (epididymal retinoic acid binding protein) fait partie de cette famille de protéines. Elle est exprimée au niveau de la tête de l'épididyme où elle représente jusqu'à 15% des protéines de ce segment. Le rôle exact de E-RABP n'est pas connu. On sait toutefois qu'elle peut se lier à l'acide rétinoïque qui est impliqué dans la régulation des fonctions épididymaires et qu'elle est nécessaire pour la survie des spermatozoïdes [60, 61].

Les protéines se liant aux lipides comme la phosphatidylethanolamine-binding protein (PEBP1) et les clusterines sont quant à elles impliquées niveau du remodelage de la membrane plasmique des spermatozoïdes. Les clusterines sont les protéines les plus fortement exprimées dans l'épididyme représentant jusqu'à 30% des protéines dans certaines régions. Elles sont toutefois rapidement réabsorbées et rarement retrouvées dans le plasma séminal [56]. PEBP1 est également un agent décapacitant [62, 63].

Les enzymes antioxydants sont aussi très importants pour la maturation des spermatozoïdes. Plusieurs de ces enzymes sont exprimés dans l'épididyme dont le glutathion peroxydase, la superoxyde dismutase et la catalase [56]. Les spermatozoïdes sont très

sensibles aux stress oxydatifs et aux espèces réactives de l'oxygène (ROS). Une trop forte concentration de ROS endommage la membrane plasmique des spermatozoïdes, diminue leur motilité et leur habileté à fusionner avec un ovocyte en plus de causer une augmentation des dommages à l'ADN pouvant entraîner le transfert de gène défectueux à l'embryon [64]. La présence de ces enzymes est donc cruciale afin de diminuer les concentrations de ROS retrouvées dans les épididymes.

Une dernière famille de protéines, représentant environ 15% des protéines de l'épididyme, est la famille des cysteine-rich secretory proteins (CRISP). Ces protéines majoritairement retrouvées dans la queue de l'épididyme se lient à la portion dorsale le la tête des spermatozoïdes et migrent vers le segment équatorial suivant la capacitation. Ces protéines sont impliquées au niveau de l'interaction spermatozoïde-ovocyte et pourraient également agir en tant qu'agents décapacitants [65-70].

4. Le plasma séminal

Lors de l'éjaculation, les sécrétions provenant des testicules et des épididymes sont mixées avec les sécrétions provenant des glandes accessoires à savoir les vésicules séminales, la prostate, les glandes bulbo-urétrales et l'ampoule. Le fluide résultant se nomme plasma séminal. Le volume total d'un éjaculat varie grandement d'une espèce à l'autre allant de 3-5 µl chez la souris à 2-6 ml chez l'humain et à plus de 500 ml chez le porc [71]. Le plasma séminal représente entre 95-98% de ce volume. Il est composé de protéines, de lipides, de fructose, de plusieurs autres types de carbohydrates et de quelques minéraux [72]. Chez l'humain, un éjaculat contient en moyenne 180 millions de spermatozoïdes [73]. Le rôle principal du plasma séminal est de fournir un milieu assurant la survie et le transport des spermatozoïdes vers le tractus génital femelle. Le plasma séminal est également riche en protéines. Chez l'humain, on estime que la concentration protéique du plasma séminal varie entre 35 et 55g/L [74]. Ces protéines sont importantes pour la complétion de la maturation des spermatozoïdes, pour la fécondation de l'ovocyte et pour le développement embryonnaire [75].

Le temps de contact des spermatozoïdes avec le plasma séminal varie d'une espèce à l'autre. Chez l'humain, suite à l'éjaculation, les spermatozoïdes entrent dans le tractus génital de la femme et, environ 30 à 90 secondes plus tard, traversent le mucus cervical se trouvant au niveau du col de l'utérus. Ce passage à travers le mucus, retire une grande partie des

composantes du plasma séminal. Le temps de contact est donc très court entre les spermatozoïdes et le plasma séminal [76]. Chez les rongeurs, le processus est différent. Le sperme est déposé dans le vagin et les spermatozoïdes sont balayés rapidement jusqu'à l'utérus avec le plasma séminal. Le contact est donc un peu plus long [36]. Ceci explique en partie pourquoi la composition et la concentration du plasma séminal varient énormément d'une espèce à l'autre.

5. La formation du réservoir oviductal

Des centaines de millions de spermatozoïdes éjaculés qui entrent le tractus génital femelle, seulement quelques milliers se rendront jusqu'à l'oviducte, le site de fécondation [77]. Chez plusieurs espèces, les spermatozoïdes ont la capacité de se lier à la paroi épithéliale de l'oviducte pour former un réservoir. Cet attachement permet aux spermatozoïdes de survivre et rester motile plus longtemps [78]. Les spermatozoïdes sont relâchés de ce réservoir au moment de l'ovulation, lorsqu'ils entrent en contact avec les composantes du liquide folliculaire expulsé des ovaires au même moment que l'ovocyte [79]. Chez les espèces bovines, des études démontrent que les protéines responsables de l'attachement des spermatozoïdes à l'épithélium de l'oviducte seraient les protéines Binder of SPerm (BSP) (section 8.3.2).

6. La capacitation

C'est également dans l'oviducte que les spermatozoïdes subissent la capacitation. La capacitation est une étape clé de la maturation des spermatozoïdes qui les rend aptes à se lier à la ZP, à subir la réaction acrosomique et à fusionner avec l'ovocyte [80]. Bien que la capacitation ait été décrite pour la première fois il y a plus de 60 ans, les mécanismes exacts de ce processus sont encore mal compris [81, 82]. On sait toutefois que plusieurs changements sont associés avec la capacitation incluant : la modification de la composition lipidique de la membrane plasmique des spermatozoïdes; l'augmentation du pH intracellulaire; l'augmentation de la perméabilité aux ions Ca^{2+}; l'activation de plusieurs voies de signalisation; l'augmentation de la phosphorylation de résidus tyrosine et le développement de la motilité hyperactive du flagelle [83-87].

La capacitation se fait de manière asynchrone. Cela assure une certaine hétérogénéité des gamètes et favorise la présence de spermatozoïdes capacités au site de fécondation. Plusieurs études ont démontré que la capacitation est temporaire et de courte durée, existant entre 50 minutes et 4 heures pour les spermatozoïdes humain, après quoi les spermatozoïdes cessent d'être capacités [88]. C'est pourquoi les facteurs décapacitants sont importants pour la fertilité [80]. Chez la souris, la protéine PEBP1 est un de ces facteurs. En plus de jouer un rôle dans le remodelage de la membrane du spermatozoïde lors du transit épididymaire, elle peut aussi se lier à cette membrane. Cette liaison permet par la suite d'inhiber la capacitation, d'empêcher la phosphorylation des résidus tyrosine et de bloquer l'interaction des spermatozoïdes avec la ZP. Chez les souris knock-out (KO) pour la protéine PEBP1, il a été démontré que les spermatozoïdes subissaient une capacitation prématurée. Par conséquent, les souris KO pour PEBP1 sont considérées comme étant sous-fertiles puisque leur nombre de souriceaux par portée est plus petit que pour des souris de type sauvage [62, 63]. Chez le bovin, les facteurs décapacitants les plus importants sont les protéines de la famille BSP [89].

La capacitation des spermatozoïdes est contrôlée par plusieurs facteurs. Trois des facteurs extrinsèques essentiels pour induire la capacitation *in vitro* sont les accepteurs de stérols (albumine et/ou lipoprotéines de haute densité (HDL)), le bicarbonate (HCO_3^-) et le calcium [80]. Les accepteurs de stérols sont tous deux retrouvés *in vivo* dans le fluide folliculaire et les sécrétions de l'oviducte. L'albumine est une protéine qui sert entre autres au transport des acides gras et qui a la capacité de se lier au cholestérol. Les HDL sont des macromolécules composées de phospholipides, de triglycérides, de cholestérol et d'apoprotéines et sont impliquées au niveau du transport du cholestérol.

6.1 Efflux de lipides

L'ordre exact dans lequel les évènements liés à la capacitation se déroulent n'est pas encore certain. L'hypothèse généralement acceptée veut que la capacitation soit premièrement induite par un efflux de stérols et de phospholipides, qui sont retirés de la membrane plasmique par l'albumine et/ou les HDL [90]. Cet efflux cause une diminution considérable du ratio cholestérol : phospholipides au niveau de la membrane plasmique, ce qui déstabilise la membrane et modifie sa fluidité [80, 91]. L'augmentation de la fluidité de la membrane permet d'augmenter son potentiel de fusion nécessaire pour la réaction de l'acrosome [92].

Plusieurs protéines retrouvées à la surface des spermatozoïdes, dont SR-BI, CD-36 et les transporteurs ATP-binding cassettes (transporteurs ABC), sont des médiateurs potentiels de l'efflux de cholestérol [93].

En plus d'augmenter la fluidité de la membrane, l'efflux de cholestérol causerait également une diminution de la taille des radeaux lipidiques et un réarrangement de ceux-ci [92]. Plusieurs études réalisées chez l'humain et la souris ont en effet démontré que, suite à la capacitation, certaines protéines disparaissent des radeaux lipidiques alors que d'autres protéines y apparaissent [92, 94, 95]. Ce réarrangement permettrait de concentrer les protéines impliquées dans la reconnaissance des gamètes au niveau du segment équatorial de la tête des spermatozoïdes et d'activer les différentes cascades de signalisation [80, 91].

6.2 Cascades de signalisation

L'activation des cascades de signalisation est également une étape très importante de la capacitation puisqu'elle mène à la phosphorylation des résidus tyrosine de plusieurs protéines. Cette phosphorylation serait entre autre responsable de l'activation des battements du flagelle, en plus de préparer certaines protéines pour la liaison à l'ovocyte et de modifier la machinerie d'exocytose permettant la réaction acrosomique [96, 97]. L'incubation de spermatozoïdes en présence d'accepteurs de stérols entraine une augmentation de la phosphorylation de manière dose-dépendante [91]. Ceci tend à démontrer que l'efflux de cholestérol précède l'activation des cascades de signalisation et la phosphorylation des résidus tyrosine. Toutefois, le lien direct entre l'efflux de stérols et la phosphorylation n'est pas encore bien compris [92].

Les cascades de signalisation impliquées dans la capacitation sont particulières puisqu'elles ne nécessitent pas la liaison spécifique d'un ligand à un récepteur [80]. L'activation des cascades de signalisation débuterait plutôt par une augmentation du pH intracellulaire causée par l'entrée de bicarbonate dans les cellules via des échangeurs Na^+/HCO_3^- [97]. Cette augmentation du pH entrainerait alors l'activation d'une enzyme, l'adenylyl cyclase soluble (sAC), causant une élévation de la concentration d'AMP cyclique (AMPc) intracellulaire [80, 97]. En plus d'une augmentation de bicarbonate dans les cellules, il est possible d'observer très tôt dans la capacitation une augmentation de calcium intracellulaire. Cette élévation du calcium serait causée par une augmentation de la perméabilité membranaire, engendrée par la perte de stérol et contribuerait également à

l'activation de sAC [98]. On attribue également l'augmentation du calcium intracellulaire à la présence de canaux calciques de la famille CatSper localisés dans le flagelle du spermatozoïde (Figure 7) [97, 99].

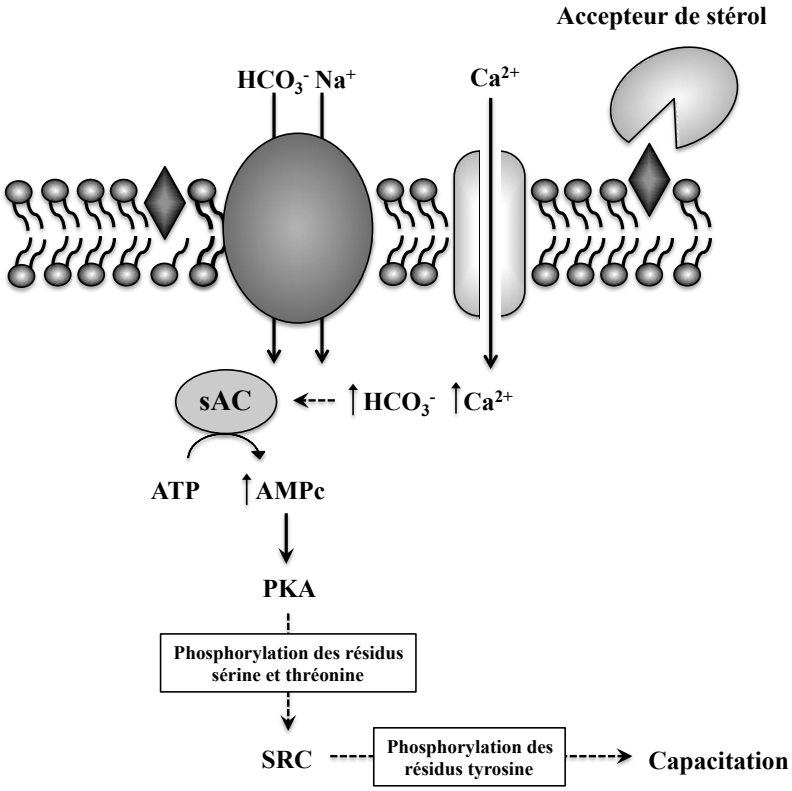

Figure 7 : Activation de la capacitation. Les accepteurs de stérol retirent le cholestérol de membrane plasmique et la déstabilisent. Cela est suivi d'une augmentation du bicarbonate intracellulaire via des échangeurs Na^+/HCO_3^- et du calcium intracellulaire via l'ouverture de canaux calciques. Ces augmentations activent sAC, ce qui augmente l'AMPc intracellulaire, active PKA, SRC et mène à la phosphorylation des résidus tyrosine. (Adapté de [97])

Plusieurs kinases impliquées dans la capacitation sont connues. La kinase la plus importante et la plus étudiée est la protéine kinase A (PKA) [97]. PKA est activée dans les spermatozoïdes par l'augmentation d'AMPc intracellulaire. Toutefois, comme PKA est une kinase à sérine et à thréonine, elle ne peut être responsable directement de la phosphorylation des résidus tyrosine [96]. Elle activerait plutôt une série de cascades de phosphorylation menant à l'activation de kinases de la famille SRC. Ce sont les protéines de cette famille qui mèneraient à la phosphorylation des tyrosines [100, 101].

Plusieurs autres voies de signalisation sont aussi activées lors de la capacitation. On compte parmi celles-ci la voie de la protéine kinase C (PKC), la voie de la protéine kinase activée par un agent mitogène (MAPK) et la voie de la phosphatidyl-inositol-3-kinase (PI3K)/Akt. Le rôle de ces différentes voies et leurs mécanismes d'activation ne sont cependant pas très bien compris.

6.3 Hyperactivation

La capacitation des spermatozoïdes est souvent associée au développement de la motilité hyperactive du flagelle. Il est important de noter que la capacitation et l'hyperactivation sont des processus distincts l'un de l'autre [102]. Ces deux événements se déroulent dans l'oviducte *in vivo* et dans les même milieux *in vitro*, requièrent les mêmes conditions et font intervenir des cascades de signalisation similaires. Toutefois, la capacitation ne requiert pas l'activation d'une motilité hyperactive et vice-versa. Il existe donc une relation temporelle entre les deux processus et non pas une relation de cause à effet [80, 97, 102, 103].

La motilité hyperactive est un patron de mouvements des spermatozoïdes qui est observé au moment de la fécondation. Ce mouvement est caractérisé par une augmentation de l'amplitude de courbure du flagelle et un battement asymétrique du flagelle [85]. Le calcium joue également un rôle important pour l'hyperactivation. Les souris KO déficientes en canaux calciques CatSper ne présentent aucune motilité hyperactivée [104]. L'hyperactivation faciliterait le détachement des spermatozoïdes du réservoir de spermatozoïdes dans l'oviducte et le passage des spermatozoïdes au travers des couches externes de l'ovocyte [97, 105].

7. Interaction des gamètes

Suivant la capacitation, les spermatozoïdes sont aptes à féconder un ovocyte. Le processus de fécondation de l'ovocyte se fait en 5 étapes (Figure 8). Les spermatozoïdes capacités sont attirés vers l'ovocyte par des chimioattractants sécrétés par les cellules du cumulus [106]. Une fois qu'un spermatozoïde a atteint l'ovocyte, il traverse le cumulus oophorus puis se lie à la ZP. L'interaction avec la ZP induit la réaction acrosomique ce qui permet au spermatozoïde de pénétrer et traverser la ZP. Lors de la dernière étape de la fécondation, les membranes plasmiques du spermatozoïde et de l'ovocyte fusionnent ce qui mène à la fusion des pronucléus pour former le zygote [107].

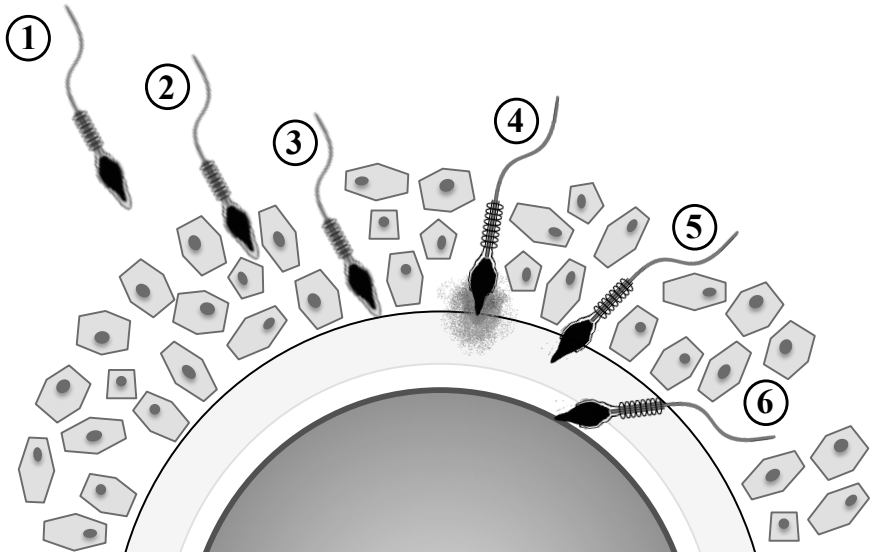

Figure 8 : Représentation schématique de la fécondation d'un ovocyte par un spermatozoïde. Le spermatozoïde subit la capacitation (1) et pénètre au travers du cumulus oophorus (2). Il se lie ensuite à la zone pellucide (3) ce qui induit la réaction acrosomique (4). Le spermatozoïde traverse finalement la zone pellucide (5) avant de fusionner avec l'ovocyte (6). (Adapté de [108])

7.1 Réaction acrosomique

Lorsque les spermatozoïdes atteignent la surface de la ZP, ils subissent la réaction acrosomique, une étape de la fécondation irréversible. Lors de la réaction acrosomique, la membrane externe de l'acrosome et la membrane plasmique du spermatozoïde fusionnent et forment des vésicules (Figure 9). Cela mène à l'exocytose du contenu de l'acrosome composé principalement d'enzymes [29]. L'enzyme majeure de l'acrosome, l'acrosine est une protéase capable de lyser la ZP permettant ainsi le passage du spermatozoïde jusqu'à l'ovocyte [109].

In vivo, la réaction acrosomique est induite par une action synergique de la glycoprotéine ZP3 et de la progestérone sécrétée par les cellules du cumulus [110]. La stimulation de la réaction acrosomique par ZP3 et par la progestérone enclenche des voies de signalisation totalement différentes. ZP3 se lie à un récepteur spécifique situé au niveau de la partie antérieur de la tête du spermatozoïde et fait intervenir des récepteurs couplés aux protéines G_1/G_0. La progestérone quant à elle agit via un récepteur de spermatozoïde semblable à celui de l'acide γ-aminobutyrique (GABA) de type A [110]. Dans les deux cas,

l'activation des signaux de transduction intracellulaire cause une augmentation du calcium dans la tête du spermatozoïde. Cette augmentation de calcium permet l'activation de plusieurs voies de signalisation faisant intervenir PKC, sAC et la Phospholipase A2 (PLA_2), ce qui mène à la dépolymérization de l'actine et à la fusion des membranes [111]. L'augmentation du calcium est causée par l'ouverture de plusieurs canaux calciques et de la mobilisation de réserves de calcium interne. Elle se fait rapidement et est visible entre 1 et 5 minutes suivant le contact avec ZP3 ou la progestérone.

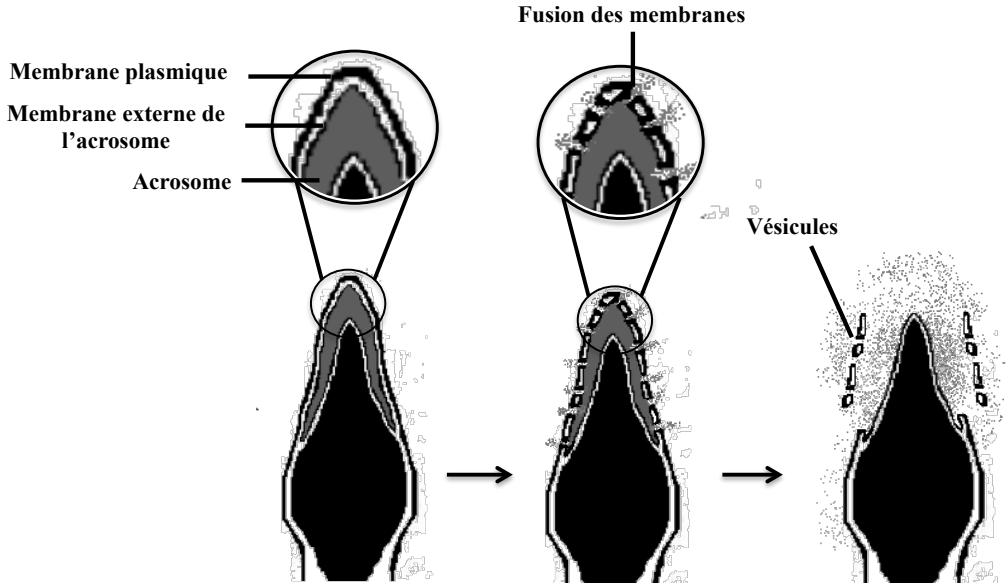

Figure 9 : Représentation schématique de la réaction acrosomique. Suite à des stimuli externes, la membrane externe de l'acrosome et la membrane plasmique du spermatozoïde fusionnent et forment des vésicules relâchant ainsi vers l'extérieur le contenu de l'acrosome. (Adapté de [112])

La réaction acrosomique peut être induite *in vitro* en incubant des spermatozoïdes capacités en présence de progestérone, de ZP solubles ou encore d'ionophores de calcium. Les ionophores de calcium sont des petites molécules capables de s'insérer dans les membranes plasmiques de cellules intactes pour permettre au calcium d'y entrer. Lors de l'induction de la réaction acrosomique à l'aide d'ionophore de calcium, plusieurs voies de signalisation normalement activées par la progestérone ou la ZP sont contournées. Cependant, dans tous les cas, la réaction acrosomique résultante est morphologiquement identique [110].

7.2 Fusion spermatozoïde-ovocyte

La dernière étape de la fécondation est la fusion des membranes du spermatozoïde et de l'ovocyte. Juste avant la fusion, les microvillosités à la surface de l'ovocyte entourent la tête du spermatozoïde [113]. C'est au niveau de ces microvillosités que débute la fusion pour l'ovocyte. Pour le spermatozoïde la fusion débute au niveau du segment équatorial de la tête [113].

Pour qu'il y ait fusion des membranes, il est tout d'abord important que le spermatozoïde adhère à l'ovocyte, ce qui implique plusieurs protéines de surface. La protéine CD9 de l'ovocyte et la protéine IZUMO du spermatozoïde font partie des protéines essentielles pour cette étape. D'autres protéines du spermatozoïde dont les protéines ADAM et les protéines CRISP1 et CRISP2 aideraient également à l'adhésion à l'ovocyte, mais ne sont pas essentielles. Les souris KO pour ces gènes ne sont pas infertiles contrairement au souris KO pour CD9 et IZUMO [113, 114].

Une fois le spermatozoïde lié à l'ovocyte, la protéine IZUMO forme un complexe avec JUNO, une autre protéine du spermatozoïde. Ce complexe permet la formation de pores de fusion au niveau des microvillosités de l'ovocyte et enclenche la fusion des deux membranes plasmiques. Une fois les membranes fusionnées, des cascades de signalisation sont activées dans le zygote pour bloquer la polyspermie [109].

8. Les protéines BSP

Depuis une trentaine d'années, notre laboratoire étudie une famille de protéines appelées protéines BSP (précédemment appelées Bull Seminal Plasma proteins) [115]. Ces protéines ont tout d'abord été identifiées chez le taureau où trois protéines BSP1, BSP3 et BSP5 (précédemment appelées BSP-A1/A2 ou PDC-109, BSP-A3 et BSP-30K respectivement) ont été isolées du plasma séminal. Elles sont exprimées par les vésicules séminales et sont de petite taille allant de 15-17 kDa pour BSP1 et BSP3 jusqu'à 28-30 kDa pour BSP5. Les protéines BSP possèdent une structure commune composée d'un fragment amino-terminal variable suivi de deux domaines de type-II semblables aux domaines de liaison au collagène de la fibronectine (Fn2), séparés par un linker de 7 acides aminés (Figure 10) [115]. Elles ne portent toutefois pas toutes les mêmes modifications post-traductionelles puisque BSP1 et

BSP5 sont glycosylées et que BSP3 ne l'est pas.

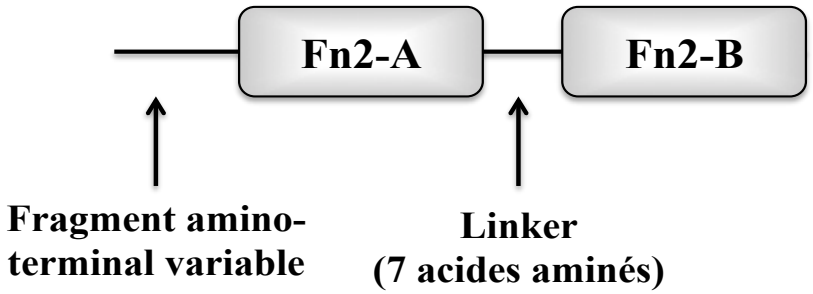

Figure 10 : Représentation schématique des protéines BSP chez les animaux de ferme.

([115])

Les protéines BSP sont ubiquitaires chez les mammifères. Suivant leur découverte chez le taureau, des protéines homologues aux BSP ont été isolées du plasma séminal ou des sécrétions des vésicules séminales de plusieurs espèces dont le bison, le buffle, le porc, l'étalon, le bélier, et le bouc [116-121]. Toutes ces protéines possèdent une structure similaire, mais varient d'une espèce à l'autre au niveau du nombre de protéines (1 à 4 selon l'espèce) et au niveau de leur concentration dans le plasma séminal qui se situe entre ~ 0.3 mg/ml chez le porc et plus de 39 mg/ml chez le taureau.

8.1 Expression et régulation

Chez les animaux de ferme, les protéines BSP sont principalement exprimées par les vésicules séminales. Chez le taureau et l'étalon, elles sont également exprimées au niveau de l'ampoule [122, 123]. Longtemps, ces deux glandes ont été considérées comme les seuls sites d'expression des protéines BSP. Toutefois, récemment, trois nouveaux gènes codant pour des protéines BSP (*BSP4*, *BSPH1* et *BSPH2*) ont été identifiés chez le bovin [124]. Étonnamment, l'expression de *BSPH1* et *BSPH2* a été détectée fortement au niveau des épididymes et légèrement dans les testicules. Des différences ont également été rapportées chez le porc, puisque l'expression de *BSP1* a été observée dans plusieurs tissus mâles incluant les épididymes (caput et corpus), les testicules et les glandes bulbo-urétrales, et dans certains tissus femelles dont le col de l'utérus et les ovaires [125, 126]. Il s'agit de la seule espèce où l'expression de gènes BSP a été détectée chez la femelle.

Peu d'informations sont disponibles sur la régulation de l'expression des gènes codants pour les protéines BSP. Plusieurs expériences réalisées suggèrent toutefois une expression régulée par la présence d'androgènes [125-127].

8.2 Partenaires d'interaction

L'étude des protéines BSP a permis de révéler que ces protéines interagissent avec une grande gamme de partenaires incluant la gélatine, la calmoduline, le HDL, ApoA-I, les lipoprotéines de faible densité (LDL), l'insulin-like growth factor II, PLA_2, les protéines de lait (micelles de caséines, α-lactalbumine, β-lactoglobuline) et les glycosaminoglycanes (GAG) comme l'héparine et le chondroïtine sulfate B (CSB).

8.2.1 La gélatine

Toutes les protéines de la famille des BSP peuvent se lier à la gélatine, un produit de l'hydrolyse partielle du collagène [128]. Chez certaines espèces, notamment chez le porc, cette interaction est plus faible [129]. Cette propriété de liaison est due à la présence des domaines Fn2 dans la structure des BSP, mais n'est, pour l'instant, directement associée à aucune fonction biologique. Elle est toutefois exploitée pour isoler les protéines BSP du plasma séminal de plusieurs espèces [118-120, 128, 130, 131].

8.2.2 Le HDL

Tel que mentionné précédemment, le HDL est une macromolécule composée de phospholipides, de triglycérides, de cholestérol et d'apoprotéines qui est importante pour le transport du cholestérol mais également pour la capacitation des spermatozoïdes. Des essais immunologiques ont démontré que, chez le taureau, BSP1, BSP3 et BSP5 sont toutes capables de lier le HDL et plus spécifiquement ApoA-I, la protéine majeure du HDL [132]. Cette interaction est nécessaire pour l'action des BSP au niveau des fonctions spermatiques.

8.2.3 Les GAG

Les GAG sont impliqués dans plusieurs étapes de la fécondation en raison de leur habileté à stimuler la capacitation chez certaines espèces, à induire la réaction acrosomique et en raison de leur implication au niveau de l'interaction entre le spermatozoïde et la ZP [133,

134]. L'interaction entre les protéines BSP et les GAG est aussi nécessaire pour l'induction de la capacitation chez le taureau [135]. Cette liaison est due à une interaction entre les acides aminés basiques des protéines et les charges négatives des GAG [136, 137]. Chez l'étalon, cette interaction est modulée par la glycosylation et l'agrégation des protéines BSP [117].

8.2.4 Phospholipides et membrane des spermatozoïdes

L'interaction des protéines BSP avec la membrane plasmique des spermatozoïdes a été largement étudiée. Chez le taureau, on a premièrement démontré que la liaison des BSP à la surface des spermatozoïdes se fait via une interaction avec les phospholipides de la membrane [138]. Les protéines BSP peuvent interagir avec plusieurs types de phospholipides, mais ont une affinité plus spécifique pour les phospholipides contenant des groupements choline dont le PC, le lyso-PC, le plasmalogène à choline et le sphyngomyéline [138]. L'utilisation de liposomes de PC et de membranes artificielles a permis de démontrer que l'interaction des BSP avec les membranes est modulée par plusieurs facteurs incluant la composition lipidique de la bicouche, le ratio lipide/protéine et la température [139]. Jusqu'à ce jour, la spécificité de liaison des BSP aux phospholipides n'a été testée qu'avec les protéines bovines. Cependant, toutes les protéines BSP provenant des animaux de ferme peuvent se lier aux liposomes de PC.

La liaison des BSP aux phospholipides portant des groupements choline permet de rigidifier et de stabiliser la membrane des spermatozoïdes de taureaux [140]. En effet, l'insertion partielle des protéines BSP dans le feuillet externe de la bicouche lipidique lors de leur liaison aux spermatozoïdes, permet de réduire la mobilité des chaines lipidiques jusqu'au $14^{ème}$ atome de carbone [141]. Cet effet stabilisateur des BSP a été démontré chez le taureau, l'étalon et le bouc [142, 143].

8.3 Fonctions

Les protéines BSP sont des protéines multifonctionnelles (Figure 11). Elles sont impliquées dans plusieurs évènements importants pour la fertilité ce qui inclut la motilité des spermatozoïdes, la formation du réservoir de spermatozoïdes et, le plus important, la capacitation des spermatozoïdes.

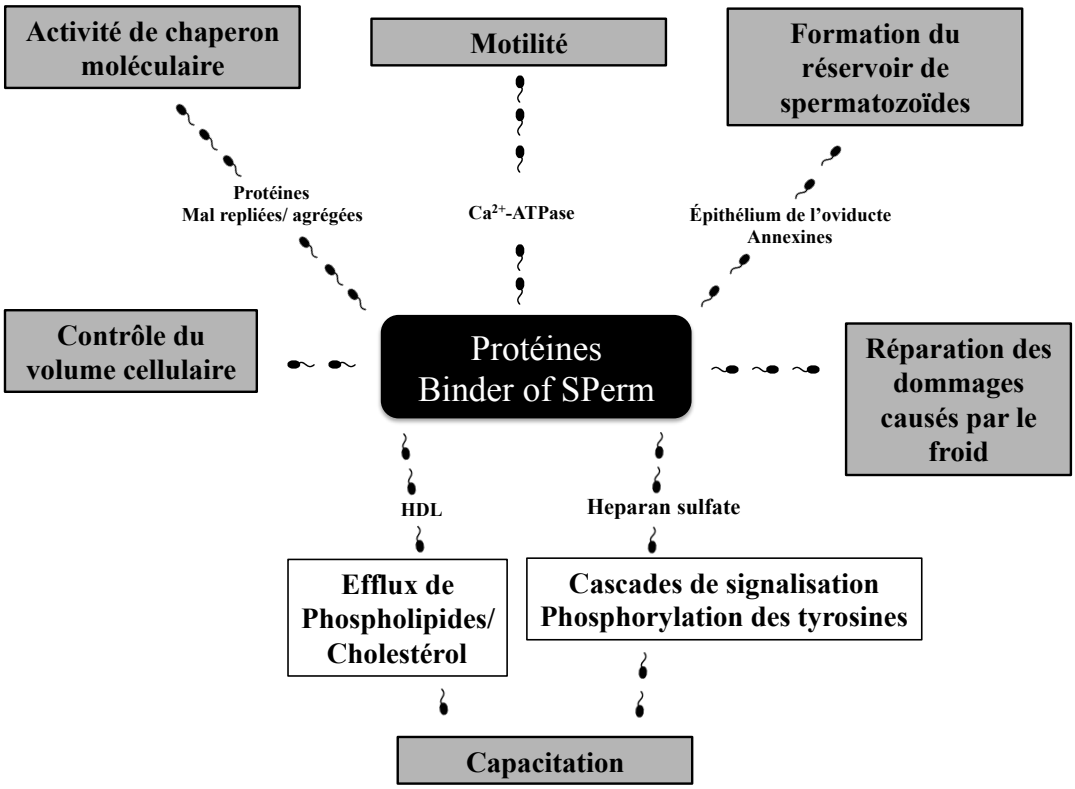

Figure 11 : Les protéines BSP sont des protéines multifonctionnelles.

8.3.1 Motilité

Les spermatozoïdes quittant les épididymes ont déjà la possibilité de se mouvoir, mais leur motilité augmente suivant l'éjaculation. Des études chez le taureau ont révélé que BSP1, qui est ajouté aux spermatozoïdes au moment de l'éjaculation, augmente la motilité des spermatozoïdes de manière significative et de manière dose-dépendante [144]. Cet effet de BSP1 sur la motilité se ferait via l'activation et/ou l'amplification de l'activité d'une pompe Ca^{2+}-ATPase [144].

8.3.2 Réservoir de spermatozoïdes

Chez le taureau, les protéines BSP sont considérées comme des facteurs importants pour la formation du réservoir de spermatozoïdes au niveau de l'oviducte. *In vitro,* l'addition de BSP1, BSP3 ou BSP5 à des spermatozoïdes épididymaires permet d'augmenter la liaison de ceux-ci à des explants d'oviducte et permet par le fait même de prolonger leur survie [145, 146]. Ces observations ont mené au modèle suivant pour expliquer la formation du réservoir

oviductal chez les espèces bovines. À l'éjaculation, les protéines BSP se lient aux spermatozoïdes et l'excès de protéines est retiré suite au passage à travers le mucus cervical à l'entrée du tractus génital femelle. Une fois dans l'oviducte, les spermatozoïdes interagissent avec l'épithélium, possiblement via une interaction entre les protéines BSP avec des annexines se trouvant à la surface des cellules épithéliales de l'oviducte. Cette liaison empêche l'initiation prématurée de capacitation et assure la survie des spermatozoïdes. Suite à l'ovulation, des facteurs du fluide folliculaire sont relâchés, détachant les spermatozoïdes du réservoir via l'activation de la motilité hyperactive. Les spermatozoïdes peuvent alors subir les étapes subséquentes de la fécondation comme la capacitation, la réaction de l'acrosome et la fusion avec l'ovocyte.

8.3.3 Capacitation

La fonction primaire associée aux protéines BSP est leur habileté à promouvoir la capacitation des spermatozoïdes. Ce rôle a été démontré chez le taureau et le porc et a été suggéré chez l'étalon, le bélier et le bouc [118, 129, 135, 147-151]. Chez le taureau, les trois protéines BSP sont capables d'induire la capacitation, mais ont besoin de HDL et/ou de GAG pour le faire [135]. Les trois protéines semblent agir en synergie sur la capacitation et n'ont pas un effet additif [135].

Le modèle d'action des BSP lors la capacitation est basé sur des expériences réalisées avec des spermatozoïdes bovins (Figure 12). Lors de l'éjaculation, les protéines BSP ont un double effet sur la membrane plasmique des spermatozoïdes. En raison de la forte concentration des BSP dans le plasma séminal, les protéines retirent des phospholipides et du cholestérol de la membrane, créant un premier efflux de cholestérol. Cet efflux se termine lors du passage des spermatozoïdes au travers du mucus cervical, lorsque le surplus de BSP libres est retiré. En même temps, une partie des BSP se lie à la surface des spermatozoïdes et stabilise la membrane protégeant ainsi les spermatozoïdes durant leur transit dans le tractus génital femelle. Une fois au niveau de l'oviducte, les HDL et les GAG qui s'y trouvent interagissent avec les BSP à la surface des spermatozoïdes. Ces interactions induisent la capacitation.

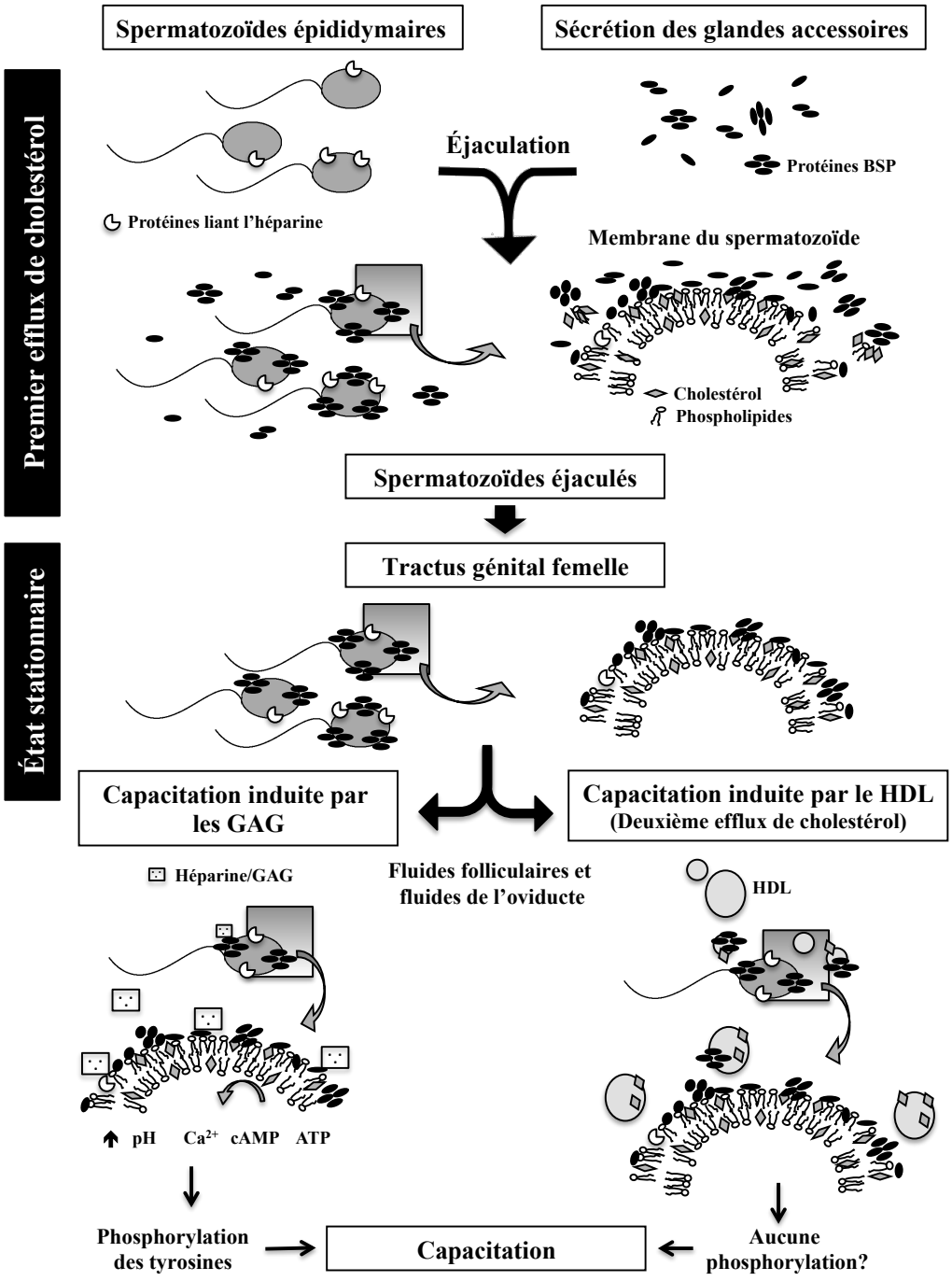

Figure 12 : Mécanisme d'action des protéines BSP au niveau de la capacitation des spermatozoïdes (Adapté de : [149, 152, 153])

Le mécanisme de capacitation induite par les HDL est différent de celui de la capacitation induite par les GAG. Comme les HDL sont des accepteurs de cholestérol, la liaison des HDL aux BSP engendre un deuxième efflux de cholestérol et de phospholipides

pour déstabiliser la membrane et débuter la capacitation. Le mécanisme exact de capacitation induite par les GAG est quant à lui inconnu. On sait par contre que l'interaction des GAG avec les BSP agirait plus au niveau de l'activation des cascades de signalisation menant à la phosphorylation des tyrosines [148, 149, 152, 154].

Plusieurs différences ont été notées entre les différentes espèces animales au niveau de l'effet des BSP sur la capacitation. Par exemple, chez le bovin, les protéines BSP liées à des spermatozoïdes épididymaires nécessitent la présence de GAG ou de HDL pour induire la capacitation. Au contraire, chez le porc, les protéines sont capables d'induire la capacitation seules sans ajout d'agents capacitants [129, 135, 147]. Chez l'étalon, les protéines BSP1 et BSP2 ne pénètrent pas aussi profondément que BSP1 de taureaux dans les membranes de phospholipides. De ce fait, elles ne peuvent pas stabiliser la membrane aussi bien et ont une capacité à extraire les phospholipides des membranes qui est plutôt faible [142]. À l'opposé, les protéines de boucs stabilisent les membranes de spermatozoïdes encore plus fortement que les protéines de taureaux [155]. Malgré ces différences, les études réalisées jusqu'à maintenant semblent toutes démontrer que les BSP jouent un rôle crucial dans la capacitation des spermatozoïdes et ce, chez tous les animaux de ferme.

8.3.4 Autres rôles

Plus récemment, plusieurs autres rôles ont été suggérés pour les protéines BSP. Des études ont démontré que BSP1 de taureaux possède une activité semblable à celle des chaperons moléculaires. Cette observation suggère que les protéines BSP pourraient aider la fécondation en dirigeant les protéines agrégées ou mal repliées du plasma séminal vers une conformation active [156, 157]. Une autre étude a montré que les spermatozoïdes épididymaires de taureaux incubés en présence de BSP1 étaient moins gonflés en réponse à des stress hypotoniques. Les auteurs de cette étude suggéraient alors un rôle des protéines contenant des domaines Fn2 dans la régulation du volume cellulaire [158].

8.4 Cryoconservation

En plus d'être importantes pour les fonctions spermatiques, les protéines BSP peuvent aussi être néfastes pour les spermatozoïdes. Chez le taureau, l'incubation de spermatozoïdes avec de fortes concentrations de protéines BSP pour une période prolongée peut causer un

efflux de cholestérol et de phospholipides continu ce qui est néfaste pour la cellule. Le lait et le jaune d'œuf sont souvent utilisés comme diluants pour la conservation du sperme. Il a été démontré que les protéines BSP peuvent interagir avec le LDL de jaune d'œuf et avec les protéines de lait [154, 159]. Ces interactions entre les protéines BSP et les composantes des diluants réduisent de manière significative la modification des membranes de spermatozoïdes résultant de l'extraction de lipides par les protéines BSP et protègent par le fait même les spermatozoïdes durant la cryoconservation [153].

Une faible quantité de BSP à la surface des spermatozoïdes semble toutefois être bénéfique pour la cryoconservation. Chez le bouc, des études ont démontré que, lors des procédures de cryoconservation, l'incubation de spermatozoïdes avec les protéines du plasma séminal avant le début du refroidissement des échantillons permet de diminuer les dommages causés par le froid. Lors de cette étude, deux protéines de bouc homologues aux protéines BSP (RSVP-14 et RSVP-22) ont été identifiées comme candidats possibles impliqués dans la réparation des spermatozoïdes suite aux dommages causés par le froid [160].

8.5 Les BSP chez l'humain et la souris

Au cours de la dernière décennie, un gène homologue codant pour une protéine BSP a été identifié chez l'humain (*BSPH1*) et deux gènes homologues ont été trouvés chez la souris (*Bsph1 et Bsph2*) [161]. La protéine BSPH1 d'humain et Bsph1 de souris sont des protéines orthologues. Elle partagent 56% d'identité et 78% de similarité de séquence [161]. Bsph2 de souris partagent quant à elle 40% d'identité et 55% de similarité de séquence avec BSPH1 et Bsph1.

Contrairement à ce qui est observé chez les animaux de ferme, ces gènes ne sont pas exprimés dans les vésicules séminales. Ils sont plutôt exprimés au niveau de la tête des épididymes humains et murins [161]. Des expériences récentes ont démontré que l'expression de *BSPH1* d'humain est vraiment restreinte à l'épididyme. Une première étude a démontré que la protéine BSPH1 chez l'humain n'est exprimée dans aucune glande accessoire, puisqu'elle n'est pas retrouvée dans l'éjaculat d'homme ayant subi une vasectomie ; une procédure qui consiste à couper le canal déférent ce qui empêche l'ajout des sécrétions testiculaires et épididymaires et l'ajout des spermatozoïdes à l'éjaculat [162]. Une seconde étude de

protéomique a confirmé en partie ces résultats en démontrant que BSPH1 ne se trouvait pas dans les testicules, les vésicules séminales et la prostate, mais était retrouvée dans le plasma séminal et à la surface des spermatozoïdes [163].

En plus d'être sécrétées beaucoup plus tôt lors du voyage du spermatozoïde au travers du système reproducteur mâle, les protéines codées par ces gènes sont retrouvées en très faible quantité dans le tractus génital mâle et possèdent une queue C-terminale absente chez les autres protéines BSP [161]. En raison de ces dissimilitudes, il était impossible de déterminer si les BSP de l'humain et de la souris possèdent des propriétés et des fonctions similaires à celles des BSP retrouvées chez les animaux de ferme, ce qui a mené au projet de recherche décrit dans cette thèse.

9. Les objectifs de la thèse

La fertilité masculine est en déclin constant depuis plus de 35 ans. Il devient donc de plus en plus important d'approfondir la compréhension des mécanismes à la base de la reproduction et d'identifier les facteurs clés de la fécondation dans le but de développer de nouveaux tests diagnostiques et de nouveaux traitements. La surpopulation de plusieurs pays et le désire de plusieurs peuples de mieux contrôler les naissances est également un enjeu mondial important. Une meilleure compréhension de la fertilité masculine pourrait aussi permettre le développement de nouveaux contraceptifs masculins pour répondre à ce problème.

Les études portant sur les protéines BSP ont démontré que ces protéines sont cruciales au niveau de la fécondation chez les bovins. La découverte de gènes codant pour des protéines de la même famille chez l'humain et la souris a ouvert de nouvelles portes vers l'identification de nouveaux facteurs ayant possiblement un impact sur la fertilité de ces espèces. Notre hypothèse de travail pour cette thèse était que les BSP chez l'humain et la souris sont ajoutées aux spermatozoïdes lors de la maturation épididymaire et ont des rôles dans les fonctions spermatiques, similaires à ceux des protéines BSP bovines. L'objectif global de cette thèse visait donc à déterminer quels rôles les protéines BSP d'humains et de souris jouent au niveau de la fertilité de ces espèces et à déterminer si ces protéines sont aussi essentielles que chez le taureau. Pour essayer de répondre à ces questions, cet objectif global a été divisé en cinq sous-objectifs.

1) Produire des protéines recombinantes BSP humaines et murines pures et fonctionnelles.
2) Tester la liaison des protéines recombinantes aux ligands connus des protéines BSP.
3) Étudier les patrons de liaison des protéines recombinantes à la surface des spermatozoïdes.
4) Vérifier l'effet des protéines recombinantes sur la motilité, la capacitation et la réaction acrosomique des spermatozoïdes d'humains et de souris.
5) Vérifier l'effet d'anticorps et de fragments d'anticorps spécifiques contre la protéine BSPH1 de souris sur la capacitation induite par la BSA et la capacitation induite par le HDL.

Article 1

Characterisation of recombinant murine Binder of SPerm protein homolog 1 and its role in capacitation.

Geneviève Plante, Isabelle Thérien et Puttaswamy Manjunath. Publié dans *Biology of Reproduction* (2012) **87**(1):20, 1-11.

Résumé

La capacitation des spermatozoïdes est une étape de maturation essentielle pour la fécondation d'un ovocyte. Une famille de protéines, les protéines Binder of SPerm (BSP), se lient aux phospholipides portant des groupements choline sur la membrane des spermatozoïdes et promeuvent la capacitation chez les espèces bovines et porcines. Récemment, des gènes homologues aux BSP ont été identifiés dans les tissus épididymaux de l'humain (*BSPH1*) et de la souris (*Bsph1*, *Bsph2*). Le but de la présente étude était de caractériser les propriétés de liaison de la protéine Binder of SPerm protein homolog 1 (BSPH1) de souris et de tester son effet sur la capacitation des spermatozoïdes murins. Puisque la purification de protéines natives BSP humaines et murines en quantité suffisante n'est pas possible, une protéine recombinante BSPH1 (rec-BSPH1) a été produite et utilisée pour les études fonctionnelles. Comme les protéines BSP des espèces précédemment étudiées, rec-BSPH1 peut se lier à la gélatine, l'héparine, aux liposomes de phosphatidylcholine et aux spermatozoïdes. La protéine native BSPH1 et rec-BSPH1 ont été détectées sur la tête et sur la pièce médiane du spermatozoïde. Toutefois, le signal au niveau de la pièce médiane était plus fort suite à l'incubation des spermatozoïdes en présence d'albumine de sérum bovin. Finalement, la protéine rec-BSPH1 est capable d'induire la capacitation des spermatozoïdes, mais est incapable d'induire la réaction acrosomique. Ces résultats démontrent que la protéine épididymaire BSPH1 de souris partage plusieurs caractéristiques biochimiques et fonctionnelles avec les protéines BSP provenant des vésicules séminales des ongulés. Ceci suggère donc un rôle similaire au niveau des fonctions spermatiques.

Contribution

Pour cet article, j'ai contribué à environ 50% du développement conceptuel de l'article. J'ai choisi les expériences à faire pour l'article et j'ai construit l'article à partir des résultats obtenus. J'ai réalisé la majorité des expériences décrites dans cet article scientifique (environ 80-85%). Dr Isabelle Thérien a réalisé une partie des expériences de capacitation et d'immuno-localisation. J'ai effectué l'analyse et l'interprétation de tous les résultats obtenus. Finalement, j'ai rédigé l'article et effectué les corrections demandées lors de l'évaluation par les pairs.

Characterisation of recombinant murine Binder of SPerm protein homolog 1 and its role in capacitation[1]

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Short title*:* Role of mouse rec-BSPH1 in sperm capacitation

Summary sentence: Recombinant murine Binder of SPerm protein homolog 1 has similar binding characteristics as other proteins from the BSP superfamily and can promote sperm capacitation but not the acrosome reaction.

Key words: epididymal protein / binder of sperm (BSP) proteins / fibronectin (Fn)2 domains / recombinant protein expression / murine sperm capacitation

ABSTRACT

Sperm capacitation is a maturation step that is deemed to be essential for sperm to fertilize an oocyte. A family of proteins, the Binder of SPerm (BSP), are known to bind choline phospholipids on sperm membranes and promote capacitation in bulls and boars. Recently, BSP-homologous genes have been identified in the epididymal tissues of human (*BSPH1*) and mouse (*Bsph1, Bsph2*). The aim of this study was to determine the binding characteristics of the murine Binder of SPerm protein homolog 1 (BSPH1) and evaluate its effects on sperm capacitation. Since it is not possible to purify the native BSP proteins from human and mouse in sufficient quantity, a murine recombinant BSPH1 (rec-BSPH1) was produced and used for the functional studies. Similarly to BSP proteins from other species, rec-BSPH1 bound to gelatine, heparin, phosphatidylcholine liposomes and sperm. Both native BSPH1 and rec-BSPH1 were detected on the head and the midpiece region of sperm although a stronger signal was detected on the midpiece region when sperm were incubated in a capacitating media containing BSA. More importantly, murine rec-BSPH1 was able to capacitate sperm but was unable to induce the acrosome reaction. These results show that murine epididymal BSPH1 shares many biochemical and functional characteristics with BSP proteins secreted by seminal vesicles of ungulates and suggest that it might play a similar role in sperm functions.

INTRODUCTION

Testicular sperm released into the seminiferous tubule lumen do not have the ability to fertilize an oocyte. They acquire this ability gradually during their transit through the epididymis, via interactions with components of accessory glands secretions following ejaculation and during their journey in the female genital tract. One of the key steps of sperm maturation is the capacitation. It is broadly defined as a series of biochemical and physiological modifications, which take place in the female genital tract and render the sperm competent to fertilize an egg [1, 2]. These modifications include 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 signalling proteins [3-7].

Capacitation can be induced in vitro by incubating sperm in defined capacitation media. The most commonly used media contain all the essential components for sperm capacitation including bovine serum albumin (BSA), calcium and bicarbonate. BSA has been shown to be a critical component of in vitro capacitation as it removes cholesterol from the sperm plasma membrane and induces protein phosphorylation and calcium influx [8, 9].

The Binder of SPerm (BSP) proteins (called Bovine Seminal Plasma proteins prior to the new nomenclature [10]) have been thoroughly studied in the last 25 years. They were first identified in the bovine seminal plasma where the three BSP proteins (BSP1, BSP3 and BSP5, previously called PDC-109 or BSP-A1/A2, BSP-A3 and BSP-30K, respectively) represent approximately 60% of the total seminal plasma proteins [11-13]. These proteins, secreted by the seminal vesicles, can bind to sperm via an interaction with choline phospholipids and promote capacitation induced by glycosaminoglycans (GAGs) and high-density lipoproteins (HDL) [14-17]. Recently, bovine BSP proteins were attributed other functions including the mediation of sperm binding to the oviductal epithelium, and the ability to prolong sperm survival and motility in the oviduct [18]. Studies also reported a chaperone-like activity for BSP1 [19].

BSP proteins were identified in the seminal plasma of other species such as boar, ram, goat, stallion and bison [20-25]. These proteins are structurally similar to Bovine BSPs as they are all composed of a variable N-terminal domain followed by two fibronectin type-II (Fn2)

domains arranged in tandem [10]. They also share many biochemical and functional characteristics such as their capacity to bind to gelatine, GAGs, HDL and low-density lipoproteins, and their ability to promote sperm capacitation [17, 22-24, 26, 27].

The BSP Fn2 domains have special features which set them apart from other Fn2 domain containing proteins such as fibronectin, blood coagulation factor XII and 72 kDa and 92 kDa type IV collagenases [28]. These features were used to identify new BSP-related sequences in the genome of different species [28]. Two BSP-homologous genes in mouse (*Bsph1, Bsph2;* accession numbers DQ227498, DQ227499) and one in human (*BSPH1*; accession number DQ227497) were identified [28]. As opposed to the other members of the BSP family, human and mouse proteins were found to be expressed exclusively in the epididymis [29]. Furthermore, human BSPH1 represents only a small quantity of the total seminal plasma proteins [29].

BSP proteins play an important role in the capacitation of bovine and porcine sperm [15-18, 26, 30, 31]. It is possible that the human and mouse homologs would also be implicated in sperm capacitation. The aim of this study was to determine the binding characteristics of the murine Binder of SPerm protein homolog 1 (BSPH1) and evaluate its effects on sperm capacitation. Since it is not possible to purify native BSP proteins from mouse in sufficient quantity, a recombinant murine BSPH1 (rec-BSPH1) was expressed, purified and used for the functional studies. Murine BSPH1 is orthologous to the human BSPH1. Therefore, the study of BSP proteins using a mouse model could give a new insight on the functions and the importance of the human BSPH1 protein in male fertility.

MATERIALS AND METHODS

Animals

Pathogen-free ICR outbred mice derived from animals from Charles River Laboratories (Wilmington, MA, USA) were purchased from Harlan Laboratories Indianapolis (IN, USA) and were kept in the animal care facility of the research center. Studies were approved by the Maisonneuve-Rosemont Hospital ethics committee and mice were treated according to the guidelines of the Canadian Council of Animal Care.

Cloning of cDNA sequence into the expression vector

For the expression of the N-terminal His-tagged BSPH1, murine epididymal cDNA was used as template for Polymerase Chain Reaction (PCR) amplification of *Bsph1*. To clone the *Bsph1* coding sequence in a pET15b vector (Novagen, EMD Biosciences, La Jolla, CA, USA), the following oligonucleotide primers were used: Bsph1-F1 (*BamHI*) 5'-CGC GCT AGC CAA GTA GAA GAT TAT TAT GCA CC-3' and Bsph1-R1 (*NheI*) 5'-GCG GCT AGC CAA GTA GAA GAT TAT TAT GCA CC-3'.

For the expression of BSPH1 fused to a thioredoxin-$(His)_6$-S-tag, the pET15b-*Bsph1* vector was used as template. The following oligonucleotide primers were used to allow the sub-cloning in the pET32a expression vector (Novagen): Bsph1-F2 (*NcoI*) 5'-GTC GCC ATG GCC TTT CAA GTA GAA GAT TAT TAT GCA CC-3' and Bsph1-R2 (*BamHI*) 5'-CGC GGA TCC TCA CTC TAT ACA ATA TTT CCA AAC-3'. The PCR was done using a *pfu* polymerase (Fermentas, Burlington, ON, Canada) under the following cycles: initial denaturation at 94°C for 3 min, 33 cycles of 94°C, 45 s; 60°C, 45 s; 72°C, 1 min and a final elongation step of 72°C for 7 min. *Taq* polymerase (GE Healthcare, Baie d'Urfé, QC, Canada), was added before the final elongation step to allow sub-cloning in a pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). The *Bsph1* coding sequence was then extracted from the pCR2.1 vector and the pET32a expression vector was linearized using the proper restriction enzymes (England BioLabs, Beverly, MA, USA). Digested products were run on agarose gel, purified using Qiaex II gel extraction kit (Qiagen, Mississauga, ON, Canada) and ligated overnight using T_4 DNA ligase (Invitrogen). Ligation reactions were transformed into competent DH5α cells, and plasmid DNA was isolated using the QIAprep spin miniprep kit (Qiagen). Sequences were confirmed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Protein expression in E.coli

The positive plasmids were transformed in Origami B (DE3)pLysS competent cells (Novagen) using standard methods. Transformed cells were then plated on Luria–Bertani (LB)-agar plates containing 100 μg/ml of ampicillin (Sigma–Aldrich, Oakville, ON, Canada) and incubated at 37°C overnight after which single colonies were inoculated in liquid LB medium containing the same concentration of ampicillin. For the protein expression, 250 ml of

LB medium containing the same antibiotic was inoculated with 1/100 volume of overnight culture and bacteria were incubated at 37°C with shaking at 200 rpm until $O.D_{600nm}$ reached 0.6-0.8. To induce the expression, IPTG (Invitrogen) was added to the cell culture to a final concentration of 1 mM and the cells were incubated at 15°C, 200 rpm for 16 h. Following the induction, cells were harvested by centrifugation at 6000 × *g* for 10 min at 4°C.

Immobilized metal ion affinity chromatography (IMAC) and refolding

Cell pellets were resuspended in B-Per bacterial protein extraction reagent (Pierce, Rockford, IL, USA) as described by the manufacturer and subjected to sonication (five cycles of 10 s on ice). One volume of 4X binding buffer (2 M NaCl, 80 mM Tris-HCl, 20 mM imidazole pH 7.4) was added to the cell lysate. Urea was added to have a final concentration of 6 M and the volume was adjusted with water to four times the initial volume. The cell extract was finally centrifuged at 25 000 × *g* for 30 min to separate the soluble and insoluble fractions. The soluble fraction 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 Ni^{2+} and equilibrated with 1X binding buffer containing 6 M urea at a flow rate of 24 ml/h. The column was then washed with 5 bed volumes of 1X binding buffer and 5 bed volumes of washing buffer (500 mM NaCl, 20 mM Tris-HCl, 80 mM imidazole, 6 M urea, pH 7.4). The refolding of the bound proteins was done on-column with a decreasing urea gradient (6 M to 0 M) in 1X binding buffer (modified from [32]). Finally, the refolded proteins were eluted with three successive elution buffers containing different imidazole concentrations (500 mM NaCl, 20 mM Tris-HCl, pH 7.4 containing 100, 200 and 400 mM imidazole respectively). Based on optical density of fractions, similar quantities of all samples were precipitated with TCA (final concentration 15%) and analyzed by Sodium dodecyl sulphate-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) [33]. Trx-His-S control was prepared in the same way using Origami B (DE3)pLysS containing empty pET32a vectors and were purified by IMAC in the absence of urea.

Protein electrophoresis and western blotting

SDS–PAGE was performed according to the method of Laemmli [34] in 12 or 15% gels using the Mini-Protean 3 apparatus from Bio-Rad (Mississauga, ON, Canada). Gels were either stained with Coomassie Brilliant Blue R-250 (BioRad), or transferred electrophoretically to Immobilon-P 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 against a synthetic peptide corresponding to the last 15 C-terminal amino acids of the deduced sequence of BSPH1, affinity-purified antibodies against $(His)_6$-tagged recombinant BSPH1 (described below), or affinity-purified antibodies against bovine BSP1 at concentration of 1:1000 [17]. 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 (Perkin–Elmer, Boston, MA, USA) and a Fuji LAS-3000 image analyzer (Fujifilm; Stamford, CT, USA).

Generation of antibodies against BSPH1

The polyclonal antibodies against murine BSPH1 were produced as described previously by Lefebvre et al. [35]. Briefly, a 15 amino acids peptide corresponding to the C-terminus of murine BSPH1 (SLTPNYNKDQVWKYC) was synthesized and conjugated to keyhole limpet hemocyanin at the Sheldon Biotechnology Center (Montreal, QC, Canada). New Zealand rabbits were injected hypodermically with a homogeneous mixture of 200 µg conjugated peptide dissolved in 100 µl of 50 mM phosphate-buffered saline (PBS), 400 µl of sterile 0.9% NaCl and 500 µl of Freund's complete adjuvant (Sigma-Aldrich). Boosts were performed at 20-day intervals over a period of 88 days, with 200 µg of the same antigen in the mixture, except using Freund's incomplete adjuvant instead of complete adjuvant (Sigma-Aldrich). Bleedings were performed 15 days after each injection. Antiserum from the third boost was used for the present study (anti-BSPH1).

For the production of antibodies against the $(His)_6$-tagged full length recombinant BSPH1 (anti-rec-BSPH1), the protein was expressed as described earlier and the inclusion bodies were isolated according to the instructions in the Novagen protocol handbook. For each injection, ~175 µg protein equivalents were run on a 10% SDS–PAGE gel. Fifteen micrograms of protein were run in another lane, which was cut from the gel and stained with Coomassie Brilliant Blue. After staining, the gels were aligned and the portion of the unstained

gel corresponding to BSPH1 protein was excised. Gel slices were minced in 1 ml saline solution and homogenized using a polytron. An equal volume of Freund's complete (first injection) or incomplete (subsequent injections) adjuvant was added and the solution was emulsified. New Zealand rabbits were immunized as described earlier. Antiserum from the third boost was used for the present study. Rabbits were treated in accordance with the guidelines of the Canadian Council of Animal Care.

Both antisera were passed through a Protein-A-Sepharose column to isolate the antibodies. Using the same concentration (1:1000), both antibodies gave similar results in western blots. Pre-immune rabbit serum was also passed through a Protein-A-sepharose column to purify IgG antibodies used as control (IgG).

For the inhibition of capacitation assays, anti-rec-BSPH1 antibodies were further purified on a rec-BSPH1-Affi-gel 15 column. Rec-BSPH1 proteins were coupled to Affi-Gel 15 resin (Bio-Rad) as described by the manufacturer. The antibodies isolated in the previous step were passed on the column and bound antibodies were eluted using PBS containing 0.1 M glycine (pH 2.5).

Affinity-chromatography

All operations were carried out at 4°C. Heparin-Sepharose CL-6B resin was purchased from Amersham Biosciences (Baie d'Urfé, QC, Canada). The coupling of gelatine to agarose beads was performed as previously described [13]. Chondroitin sulphate B (Sigma-Aldrich) was coupled to Affi-gel 15 as previously described [17] with the exception that 4 mg of commercial chondroitin sulphate B was coupled to 20 ml of resin. For each experiment, 5 ml of resin was packed in a column (1 cm × 15 cm) and equilibrated with 50 mM Tris-HCl buffer, pH 7.4 (TB). Since rec-BSPH1 is barely soluble in high concentrations, diluted solutions were used. 500 µg of rec-BSPH1, or control Trx-His-S 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. The unbound material was then washed from the column with TB and the column was successively washed with TB containing 1 M NaCl and TB containing 8 M urea. For the gelatine-agarose affinity chromatography, the column was washed with TB and eluted directly with TB containing 8 M urea. The protein fractions from each peak were pooled and approximately 3 µg of protein from each peak were precipitated with TCA and analyzed by SDS-PAGE. For

quality control, alchohol precipitates of bovine seminal plasma proteins were run similarly on each column.

Binding to phosphatidylcholine (PC) liposomes

PC liposomes were prepared as previously described [14]. Briefly, 10 mg of PC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Avanti Polar Lipids inc., Alabaster, AL, USA) were evaporated under N_2 to form a thin film at the bottom of a glass test tube. 2 ml of buffer A (10 mM Tris-HCl, 100 mM KCl, pH 7.5) was added to the tube and sonicated in a Branson ultrasonic water bath (model 3510) for approximately 20 min at room temperature. **small unilamellar** liposomes were sedimented at 100 000 × *g* for 30 min at 25°C and resuspended in 1 ml of buffer B (10 mM Tris-HCl, 100 mM KCl, 2.5 mM $CaCl_2$, pH 7.5). To study the binding of rec-BSPH1 (20 µg), thioredoxin (20 µg) or bovine BSP1 (10 µg) to PC liposomes, the indicated amount of proteins was incubated with 300 µg of liposomes in 300 µl of buffer B for 40 min at room temperature. Liposomes were then sedimented at 100 000 × *g* for 45 min. An equal volume of supernatant and pellet was treated with TCA (final concentration 15%) and precipitated proteins were analyzed by western blot.

Preparation of sperm

Cauda epididymides and vas deferens were removed immediately after male mice (>12 weeks old) were sacrificed by cervical dislocation. Epididymides were minced in 1 ml pre-warmed modified Krebs-Ringer medium (Whitten's HEPES (WH) buffered medium) and placed at 37°C for 10 min to allow motile sperm to swim-out [36]. Motile sperm were then collected, washed once with 5 ml of WH media (10 min at 200 × *g*) and resuspended in 500 µl of WH media.

Binding to sperm

To study the binding of rec-BSPH1 to mouse sperm, 10×10^6 sperm were incubated 1 h at 37°C in the presence or the absence of 10 µg of rec-BSPH1 in 1 ml of WH media containing Complete Mini, EDTA-free protease inhibitor tablet (1 tablet/10 ml; Roche, Manheim, Germany) and 1 mM of PMSF. Following the incubation, the sperm were pelleted at 5000 × *g* for 10 min. The supernatant was removed and the pellet was washed three times

with 1 ml of WH medium. The supernatant and wash fractions were precipitated with TCA (15%, final concentration), resuspended in Laemmli sample buffer and boiled 10 min. The pellet was resuspended in sample buffer, boiled 10 min and sonicated 1 h in a Branson ultrasonic water bath (model 3510). The different fractions were analyzed by SDS-PAGE and western blot.

Capacitation / inhibition of capacitation assay

Sperm were collected as described above. For capacitation studies, 2×10^6 washed sperm were incubated 1 h at 37°C in 1 ml WH media supplemented or not with BSA (5 mg/ml), in the presence of different concentration of rec-BSPH1, Trx-His-S or without protein as control.

For inhibition experiments, washed sperm (2×10^6) were incubated in WH media supplemented or not with 5 mg/ml BSA in the presence of different concentrations of the anti-rec-BSPH1 antibodies or IgG isolated from normal rabbit serum as control.

Following the incubation with the antibodies or recombinant proteins, 200 µl of the sperm suspension were incubated an additional 30 min at 37°C with or without 5 µM of calcium ionophore A23187 (Sigma-Aldrich). Sperm were then fixed with 200 µl of 8% paraformaldehyde for 30 minutes at room temperature, centrifuged for 2 min at 8000 × *g* and washed 2 times with 0.1 M ammonium acetate (pH 9.0). They were finally resuspended in a final volume of 100 µl of the same solution and 20 µl were smeared on microscopic slides.

Slides were then dried and stained using the Coomassie Brilliant Blue staining technique [37]. Briefly, the slides were put successively in water, methanol and water for 5 minutes each and stained 2 minutes in a solution of 0.22 % Coomassie Blue G-250 in 50% methanol and 10% acetic acid solution. 400 sperm were counted for each condition.

Chlortetracycline (CTC) staining was slightly modified from that described by Ward and Storey [38]. After incubation with the different concentrations of rec-BSPH1, anti-BSPH1 or controls, 50 µl of sperm suspension were mixed with 50 µl of CTC solution (5 mM cysteine, 750 µM CTC, 20 mM Tris base and 130 mM NaCl, pH 7.8). After 30 s, the reaction was stopped using 12.5 µl of glutaraldehyde (12.5 %). Two 15 µl droplets of this mixture were placed on a slide and covered with a cover slip. The slides were kept overnight in the dark at

4°C in a humid environment. The slides were then observed with a Leitz Diaplan fluorescent microscope under blue-violet illumination (excitation at 330–380 nm, emission at 420 nm).

Immunolocalization of rec-BSPH1 on sperm

To determine which regions of the sperm binds rec-BSPH1, sperm were prepared as described for the capacitation assays. Sperm were incubated 1 h in WH media without any added proteins, with 15 µg rec-BSPH1 or 7 µg Trx-His-S as control in the presence or the absence of BSA (5 mg/ml). Following the 30 min incubation with or without calcium ionophore, 200 µl of sperm suspension were fixed with 200 µl of 4 % paraformaldehyde for 30 min at room temperature. The sperm were then washed 3 times with PBS and allowed to dry on Poly-L-lysine microscopic slides (Fisher Scientific, Ottawa, ON, Canada). Sperm were permeabilized with PBS containing 0.1 % Triton-X-100 and 0.2 % paraformaldehyde, washed 3 times with PBS and incubated for 1 h at room temperature in PBS containing 1 % BSA. Slides were then incubated 1 h at room temperature with anti-rec-BSPH1 antibodies, mouse His-probe antibodies or IgG as control at a dilution of 1:100 in PBS 0.1 % BSA, washed three times with PBS to remove excess antibodies and incubated 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) at dilution 1:200 in PBS 0.1 % BSA. All slides were then washed three times with PBS and counterstained with 5 µg/ml Hoechst 33342 (Sigma-Aldrich) for 5 min at room temperature. After three final washes with PBS, slides were mounted with DABCO 1.5% (10 ml DABCO 15% and 90 ml glycerol). Observations were done under a fluorescence microscope (Leica DMRE, Leica Microsystems) and images were captured with a digital camera (Retiga EX; QIMAGING; with OpenLab version 3.1.1 software; Open-Lab). To evaluate the different patterns of the rec-BSPH1 protein at the surface of the sperm, 400 sperm were counted for each condition.

Statistical Analysis

Data are presented as the mean ± S.D. Differences were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test using IBM SPSS statistics (version 19). A p value of < 0.05 was considered significant.

RESULTS

Expression, purification and biochemical characteristics

As seen on Figure 1A, during the purification on a His-Bind column, the presence of proteins was observed in each step of the elution with imidazole. Analysis of the peaks by SDS-PAGE under reducing conditions (Fig. 1B) revealed the presence of a protein at approximately 32 kDa, the expected molecular weight of rec-BSPH1, in every peak (E1 to E3). In the first fraction (E1), which corresponds to elution using 100 mM of imidazole, another strong band of ~28 kDa was observed. This band was not present in fractions E2 and E3 (elution with 200 mM and 400 mM imidazole respectively). However, E2 and E3 showed a faint band at 64 kDa, which was recognized by the anti-rec-BSPH1 antibodies confirming that it is a rec-BSPH1 dimer (data not shown). Analysis of the elution fractions by SDS-PAGE under non-reducing conditions showed many bands of higher molecular weight (Fig. 1C). His-Probe monoclonal antibodies (Fig. 1D) and anti-rec-BSPH1 antibodies (not shown) both recognized the 32 kDa band and all the higher molecular weight bands in western blot analysis, indicating that these bands are different oligomeric forms of the recombinant protein. The 28 kDa band recovered in the first elution (E1) was not recognized by any of the two antibodies. LC-MS/MS analysis of the 32 kDa band confirmed the identity of BSPH1 protein (Fig. 1E). The bands on the SDS-PAGE were scanned and the following proportions were obtained for E2 and E3: monomers (26 %), dimers (18 %), tetramers (10 %), pentamers (7 %) and octomers (6 %). The remaining 34 % were higher molecular weight oligomers which could not be resolved using 12 % polyacrylamide gels. Fraction E2 and E3 were pooled and used for further experiments. E1 was discarded due to the presence of contaminants.

Binding Studies

Binding of rec-BSPH1 to gelatine, heparin, chondroitin sulfate B, PC-liposomes and sperm membranes was tested. The binding to gelatine and GAGs was assessed by affinity chromatography. When rec-BSPH1 was passed on a gelatine-agarose affinity chromatography column, about 70% of the total protein did not bind to the column (Fig. 2A and B). The bound fraction was eluted with 8 M urea. On a heparin-sepharose affinity chromatography column, no rec-BSPH1 was found in the unadsorbed fraction (fraction 1) and nearly everything was eluted with 1 M NaCl and 8 M urea (Fig. 2C and D). In contrast, more than 95% of the

recombinant protein was found in the unbound fraction when chromatographed on chondroitin sulfate B affinity column. As controls, the binding of bovine BSP proteins and the Trx-His-S was also tested on the three resins used for this study (data not shown). Trx-His-S did not bind to gelatine and chondroitin sulphate B and bound only slightly to heparin (< 20 %) while bovine BSP proteins bound strongly to all three resins.

The interaction of rec-BSPH1 with PC-liposomes was evaluated by ultracentrifugation method. Figure 3A, shows that when PC-liposomes were absent from the incubation medium, all three proteins tested were found in the supernatant after centrifugation. In the presence of PC-liposomes, the majority of rec-BSPH1 was found in the pellet fraction that contained the PC-liposomes. The same result was obtained with the bovine BSP1. In contrast, the Trx-His-S remained in the supernatant fraction. The binding of rec-BSPH1 to sperm membranes was also investigated by centrifugation method. After incubation with sperm, most of the recombinant protein was found in the pellet fraction comprising sperm (bottom panel of Figure 3B). A small amount of the recombinant protein was also detected in the supernatant fraction. The same experiment was done with sperm incubated without the recombinant protein. As seen on the top panel of Figure 3B, a band of ~14 kDa, the expected molecular weight of the native BSPH1, was detected in the pellet fraction. This band was not detected in the supernatant or the wash fractions and no other bands were detected by the anti-rec-BSPH1 (against His-Tagged protein) or the anti-BSPH1 (against C-terminus peptide) antibodies.

Capacitation assay

Since only capacitated sperm can undergo the acrosome reaction (AR), capacitation was assessed by the ability of sperm to undergo the AR induced by calcium ionophore A23187. Without the addition of rec-BSPH1 or BSA, the basal level of AR in the presence or absence of A23187 was 23 ± 3 %. As seen on Figure 4A, 10 µg/ml of rec-BSPH1 was sufficient to cause a significant increase in the percentage of sperm AR induced by A23187 ionophore. Higher concentrations of recombinant protein increased slightly the percentage of AR induced by A23187 but that number reached a plateau around 41 ± 6 % with 30 µg/ml of rec-BSPH1. BSA alone stimulated the AR induced by A23187 up to 52 ± 9 %. The addition of Trx-His-S to the media did not increase the level of sperm AR induced by A23187. As seen on Figure 4B, without the addition of calcium ionophore, the level of AR obtained for each

condition tested was comparable to the basal AR level. For all the conditions tested, similar results were also observed using CTC staining instead of Coomassie Blue staining (results not shown).

Capacitation inhibition assay

Using similar methods, inhibition of capacitation by anti-rec-BSPH1 antibodies was tested. As seen on Figure 5A, increasing concentrations of anti-rec-BSPH1 antibodies caused a dose-related decrease in the percentage of sperm AR induced by A23187 in the presence of BSA. In this set of experiments, BSA alone stimulated the AR induced by A23187 up to 39 ± 3 %. The addition of 0.5 µg/ml of anti-rec-BSPH1 antibodies was sufficient to cause a significant decrease of the level of AR induced by A23187 and the addition of 8.4 µg/ml of antibodies decreased the level of AR of sperm back to the level obtained without BSA. When antibodies were added in the absence of BSA, the basal AR level was not affected and remained the same. The addition of IgG purified from normal rabbit serum had no inhibitory effect unlike anti-rec-BSPH1 antibodies. Without the calcium ionophore, for all conditions tested, levels of AR were not significantly different than the basal level of AR (Fig. 5B).

Immunolocalization of rec-BSPH1 on sperm surface

Localization of rec-BSPH1 on sperm surface was tested in the presence of rec-BSPH1, with rec-BSPH1 and BSA, and with rec-BSPH1, BSA and calcium ionophore (Fig. 6A). When caudal sperm were incubated in those conditions, permeabilized and immunostained with anti-BSPH1 antibodies and FITC-conjugated secondary antibodies, a clear signal could be observed on the head and/or the midpiece region of the sperm. Immunodetection was tested on both permeabilized and non-permeabilized sperm (data not shown) and similar results were obtained with both conditions.

Different controls, in the presence of BSA and without calcium ionophore were done (Fig. 6B). When sperm were incubated only in the presence of BSA, a faint signal could be observed on the surface of the sperm (top panel; white arrows). When sperm were incubated with rec-BSPH1 and IgG (purified from normal rabbit serum) were substituted as first antibodies, no signal was observed (middle panel). Similarly, no signal was obtained when caudal sperm were incubated with Trx-His-S and incubated with His-probe antibodies as first

antibodies (bottom panel). For all controls, sperm were counterstained with Hoechst dye and clear signals for sperm nucleus could be observed confirming the presence of sperm on the slides.

Interestingly, sperm incubated with recombinant proteins showed three different binding patterns. Some sperm had binding only on the acrosome region, some had binding over the midpiece and acrosome regions, and some had proteins only on the midpiece region (Fig. 7), (pattern A, B and C respectively). In the absence of BSA, 75 ± 5% of the sperm had binding only on the head (grey bars) and 22 ± 4% had binding on the head and the midpiece region (white bars). When BSA was added, in the presence or absence of calcium ionophore, the results were inverted. Approximately 65 ± 4% of the sperm had binding on the midpiece region and the head, and 30 ± 5% had binding only on the head. The sperm fraction with binding only on the midpiece region (black bars) was negligible for all conditions tested.

DISCUSSION

Expression, purification and biochemical characteristics

The choice of the expression system, namely *E. coli* Origami B (DE3)pLysS and pET32a vector, to produce recombinant BSP proteins has been thoroughly discussed in a previous publication [35]. With this expression system alone, a good level of rec-BSPH1 could be obtained but, some problems regarding protein aggregation, solubility and purity were observed. To resolve these issues, a purification step using on-column refolding was added. The use of 6 M urea to denature the protein aggregates followed by a decreasing gradient from 6 M to 0 M urea on column was adequate to reduce the number of high molecular weight aggregates that were initially present. The use of this purification method resulted in the presence of soluble, pure proteins with different levels of oligomerization. This is consistent with previous observations in other species such as bull, stallion, ram and boar [12, 24, 25, 39, 40].

Binding Studies

It has been established that most proteins from the Binder of Sperm family bind to heparin and some of them also bind to chondroitin sulphate B. The ability of proteins to bind GAGs is attributed to conserved patterns of basic amino acids in the protein sequences. Because heparin

is the most negatively charged GAG, it binds more strongly to ligand than other GAGs [41, 42]. The murine BSPH1 protein contains very few consensus basic amino acid sequences, which could explain why rec-BSPH1 could bind to heparin but not to chondroitin sulphate B, a less negatively charged GAG.

BSP proteins also have the ability to bind to gelatine, a property attributed to the presence of two Fn2 domains [13]. The significance of this property is currently unknown but has been exploited for the purification of BSP proteins from seminal plasma of several ungulates (bull, ram, bison, goat, stallion) [13, 22-24]. Even though rec-BSPH1 contains Fn2 domains, ~30 % of the total proteins loaded bound strongly to gelatine. Attempts to dialyse with redox system or gradual elimination of urea did not improve binding significantly. The reason for reduced binding is unknown but it could be conformation related or mostly due to the masking of gelatine binding sites following aggregation or some unknown reasons. The bulky trx in aggregated recombinant may also interfere or mask the gelatine binding sites. It is worth mentioning that the recombinant AB domain and the recombinant full length bovine BSP5 protein do not bind gelatine yet are able to promote sperm capacitation (Jois et al. Manuscript submitted). In the current study, we used rec-BSPH1 for functional studies without fractionation on gelatine-agarose column.

Another intrinsic characteristic shared among proteins of the BSP superfamily is the ability to interact with choline containing phospholipids such as PC [14, 21, 43]. Crystallography studies have shown that PC interacts with BSP proteins through a cation interaction between the quaternary amine group of the choline and a tryptophan residue of the Fn2 domains, as well as through hydrogen bonds between three tyrosine residues of the protein and phosphate groups [44]. Since those tyrosine and tryptophan residues of the Fn2 domains are conserved between the bovine BSP proteins and the BSPH1 protein sequence [29], it is not surprising to see that the murine homolog also interacts with PC liposomes.

Choline phospholipids (PC and sphingomyelin) represent ~70% of total phospholipids in murine epididymal sperm plasma membranes [45]. This could explain the binding of BSPH1 to sperm membranes as observed with bovine BSP proteins [46]. As seen on Figure 3B, native BSPH1 is found in the sperm pellet after ultracentrifugation. Incubation of sperm in the presence of rec-BSPH1 gave similar results but a stronger signal was observed. While the presence of PC and sphingomyelin in sperm membranes explains the binding of rec-BSPH1 to

sperm, rec-BSPH1 could also bind sperm via the native BSPH1 already bound to choline groups on the sperm surface (protein-protein interaction). It has been demonstrated that bovine BSP1 binds sperm via an interaction with choline phospholipids on sperm surface as well as by self-association on the sperm surface [47]. Immunolocalization studies clearly confirmed the binding of native and recombinant BSPH1 to sperm surface.

In bovine ejaculated sperm, BSP proteins have been shown to bind over the head and midpiece region of sperm [46]. In the current study, rec-BSPH1 was shown to bind to the same regions of sperm but a greater proportion of sperm had binding over the midpiece region in the presence of BSA. These results are similar to those reported with bovine BSPs. Bovine epididymal sperm incubated with BSP1 show intense fluorescence on the acrosome region; whereas, on sperm incubated with oviductal fluid, which is composed of 85% albumin, BSP1 and BSP3, are more concentrated on the midpiece region [18, 48]. Midpiece region of spermatozoa is the region containing mitochondria and is often associated with sperm motility. Since it has already been shown that bovine BSP1 stimulates sperm motility and the activity of membrane-bound calcium ATPase in bull semen [31, 49], it is likely that BSPH1 may play a role in motility as well.

Role in sperm capacitation

The main function ascribed to BSP proteins in bovine and boar is to promote sperm capacitation [26, 50]. The current study shows that rec-BSPH1 can promote capacitation and joins a list of other murine epididymal proteins with a role in capacitation such as CRISP-1 and HongrES1 [51, 52]. In order to study the effect of BSP proteins on capacitation in bovine model, washed epididymal sperm are incubated with appropriate protein concentration, the excess or unbound BSP proteins are removed by washing and sperm are reincubated for 4-5 hr with capacitation factor (heparin) [16, 31]. Since murine sperm are very fragile [53], to better suit the mouse model, the conditions used for capacitation were different than those used in bovine model. Mouse sperm were incubated in continuous presence of rec-BSPH1 to avoid additional manipulations and centrifugation steps. The physiological concentration of the BSPH1 protein in mouse epididymis are unknown but immunodetection experiments with epididymal sperm and tissues extracts suggest that the amount is quite low (unpublished). For this reason, rec-BSPH1 ranging from 1 μg/ml to 60 μg/ml was used. It is important to note

that the tag fused to the recombinant protein accounts for 53% of the total protein. Therefore, the actual BSPH1 concentrations used in the functional experiments ranged from 470 ng/ml to 28 μg/ml.

At concentrations lower than 10 μg/ml, which are probably closer to concentrations found *in vivo* AR levels induced by A23187 did not increase. It has been demonstrated with bovine sperm that in order to promote sperm capacitation, BSP proteins need HDL or excess free BSP to act as acceptors to induce lipid efflux [16, 50, 54, 55]. In a similar manner, in this study, when low concentrations are used, all the protein binds to sperm surface and none are available to remove lipids from the sperm surface. When higher concentrations are present in the media, free BSPH1 may act as acceptor to remove phospholipids and cholesterol from sperm membranes that could lead to capacitation. Thus, *in vivo,* sperm bound BSPH1 would need to interact with components of the oviductal fluid to promote capacitation (Fig. 8).

To better understand the mechanism of the native BSPH1 in sperm capacitation, the effect of antibodies on capacitation was also tested as performed in other studies [56, 57]. Without BSA, the anti-rec-BSPH1 antibodies did not decrease the AR which remained at the basal level indicating that native (endogenous) BSPH1 is not sufficient to stimulate an increase in capacitation. The effect of antibodies was then tested on BSA induced capacitation to see if anti-rec-BSPH1 could block capacitation induced with other factors. These experiments showed that 500 ng/ml anti-rec-BSPH1 antibodies were sufficient to reduce significantly the level of sperm capacitation induced by 5 mg/ml BSA. BSP proteins bind sperm surface via an interaction with the choline group of phospholipids [14] and BSA has been shown to interact with the acyl chain of phosphatidylcholine [58]. Anti-rec-BSPH1 antibodies could inhibit BSA induced capacitation by binding native BSPH1 on sperm surface preventing BSA from acting to remove sterols from sperm membranes. Bovine BSP proteins are known to coat the sperm surface, prevent free movement of the phospholipids and stabilize sperm membrane [59, 60]. Another explanation could be that, in a similar manner, binding of antibodies to the native BSPH1 on the sperm surface possibly created a stable network between BSPH1 proteins, thereby preventing movements of the lipids and further stabilizing the sperm membrane, rendering capacitation induced by the BSA impossible.

Murine BSPH1 differs from other BSP proteins as it is secreted by the epididymis and is found in low concentrations. Since epididymal maturation is a long process, it is likely that the

expression of low concentration of BSPH1 prevents detrimental effects that BSP proteins could exert. Instead, during epididymal maturation, BSPH1 proteins could be binding to sperm surface to stabilize the membrane and prevent premature capacitation and AR. Following ejaculation and the journey through the female genital tract BSPH1 could interact, in the oviduct, with HDL or other factors to promote sperm capacitation (Fig. 8).

In conclusion, a suitable method for the expression and the purification of rec-BSPH1 was developed to produce soluble and less aggregated proteins. The recombinant protein showed similar binding properties as other BSP proteins. This study is the first to demonstrate the implication of an epididymal protein from the BSP superfamily in capacitation and the first to assess the role of a BSP from rodents in sperm function. Furthermore, it is the first study using recombinant full length BSP proteins to assess sperm function. Further work to understand the exact mechanism by which mouse BSPH1 could promote sperm capacitation via its interaction with oviductal fluid components is needed. Other putative functions such as sperm-egg interaction also warrant investigation. Finally, the role of the second murine BSP protein homolog (BSPH2) in sperm functions also requires to be studied. In view of the fact, murine BSPH1 shares a high level of sequence identity with the human epididymal BSP homolog 1 (BSPH1), this study could also help to better understand the role of the BSP protein in human.

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FIGURE LEGENDS

Fig. 1. *Expression, purification and on-column refolding of rec-BSPH1*. (A) IMAC profile for the stepwise elution of rec-BSPH1 protein. Column fractions were analyzed by 12% SDS-PAGE in the presence (B) or absence (C and D) of β-mercaptoethanol. The gels were Coomassie-stained (B and C) or analyzed by western blot with His-probe antibodies (D). 0h: before protein induction; P: cell extract pellet; I: input; FT: flow through; NA: nonadsorbed; W: wash, E1 (tubes 7-16): elution with 100 mM imidazole; E2 (tubes 26-47): elution with 200 mM imidazole; E3 (tubes 55-62): elution with 400 mM imidazole. For gels in B and C, ~15 µg proteins were loaded in each lane except lanes E1-3 where 7 µg proteins were loaded and lane. For gel D, ~3 µg proteins were loaded in each slot. The pellet (P) of the cell lysate, was resuspended in sample buffer and 1/100 of the fraction was loaded in lane P. (E) LC-MS/MS analysis of the 32 kDa band in pooled E2-E3 fractions. Bold letters represent residues identified by LC-MS/MS.

Fig. 2. *Binding of rec-BSPH1 to gelatine and GAGs.* Affinity chromatography profiles and Coomassie-stained SDS-PAGE patterns of column fractions for rec-BSPH1 on gelatine-agarose (A, B), heparin-sepharose (C, D) and chondroitin sulfate B-agarose (E, F) columns. Approximately 500 µg of proteins were loaded on each column. The columns were then washed with 25 mM TB, and eluted with TB containing 1 M NaCl (GAGs columns only) and 8 M urea (GAGs and gelatine columns).

Fig. 3. *Binding of rec-BSPH1 to PC liposomes and mouse caudal sperm*. (A) 20 µg of rec-BSPH1, 20 µg Trx-His-S and 10 µg of bovine BSP1 were incubated with or without 300 µg of PC liposomes and then centrifuged. The supernatant (S) and pellet (P) were separated and analyzed by western blot using mouse anti-rec-BSPH1, His-probe and bovine anti-BSP1 antibodies respectively. (B) Caudal sperm were incubated with (bottom panel) or without (top panel) 10 µg of rec-BSPH1 and then centrifuged. The supernatant (S) was removed and the sperm pellet was washed. The wash fraction (W) and the sperm pellet (P) were separated by centrifugation and equivalent proportions of each fraction were analyzed by western blot using mouse anti-rec-BSPH1 antibodies.

Fig. 4. *Effect of rec-BSPH1 on murine sperm capacitation.* Capacitation was assessed by the ability of sperm to undergo the AR induced by A23187 ionophore. Epididymal sperm were incubated with different concentrations of rec-BSPH1 or 32 µg/ml of Trx-His-S in the

presence or absence 5 mg/ml of BSA for 60 min followed by incubation with (A) or without (B) calcium ionophore A23187 for 30 min. Sperm were smeared on slides and analyzed by Coomassie Blue staining. A minimum of 400 sperm per conditions were evaluated. Data are means ± SD for four independent experiments. *: significant difference compared to control without BSA and rec-Bsph1 ($P < 0.05$).

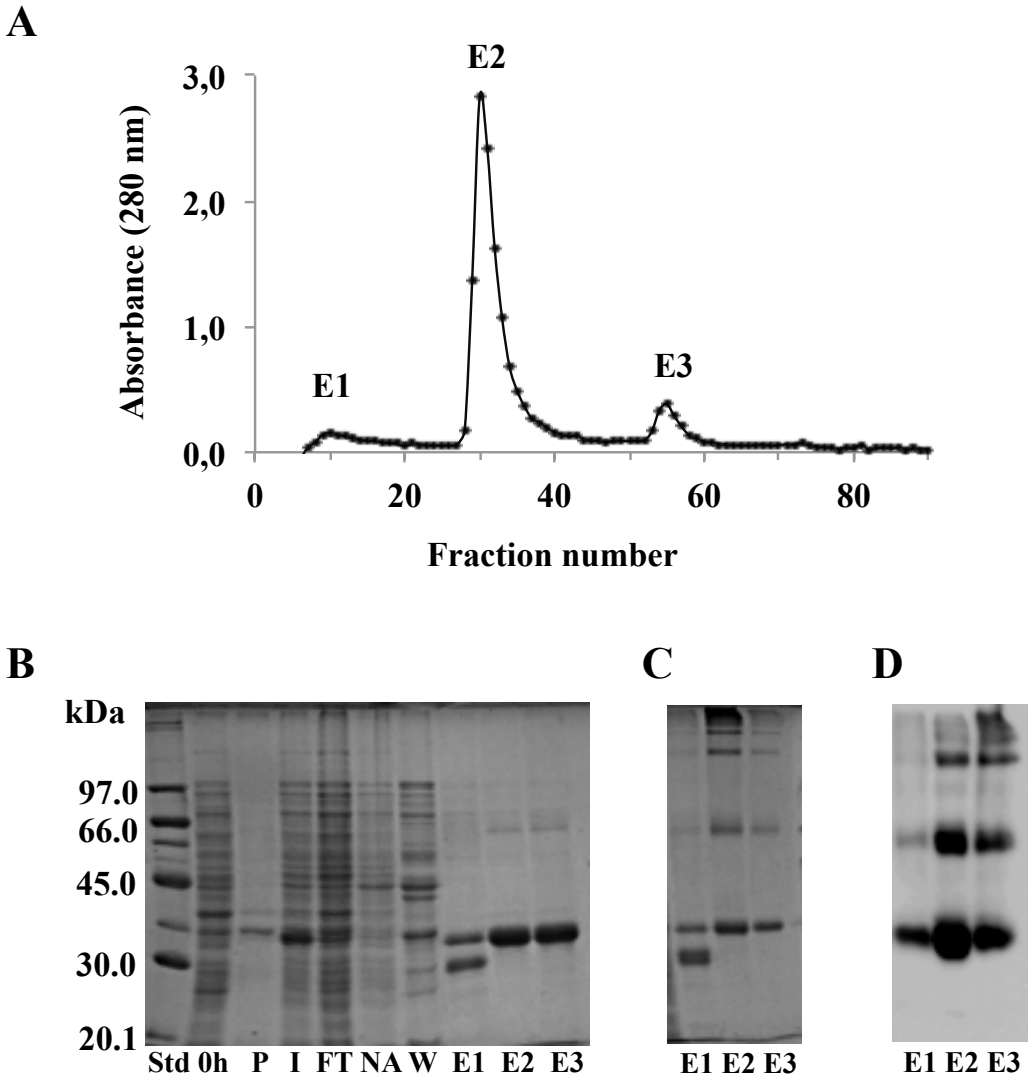
Fig. 5. *Effect of anti-rec-BSPH1 on murine spermatozoa capacitation.* Capacitation was assessed by the ability of sperm to undergo the AR induced by A23187 ionophore. Epididymal sperm were incubated with or without 5 mg/ml of BSA and different concentrations of anti-rec-BSPH1 antibodies or control IgG for 60 min followed by incubation with (A) or without (B) calcium ionophore A23187 for 30 min. Sperm were smeared on slides and analyzed by Coomassie Blue staining. A minimum of 400 sperm per conditions were evaluated. Data are means ± SD for four independent experiments. *: significant difference compared to BSA alone (control, $P < 0.05$).

Fig. 6. *Immunostaining of rec-BSPH1 on cauda epididymal sperm.* (A) Sperm collected from cauda epididymis were incubated alone or in media containing 15 µg rec-BSPH1 and BSA with or without A23187. Slides were incubated with anti-rec-BSPH1 antibodies at dilution of 1:100 and treated with FITC-conjugated IgG. (B) For detection of native proteins, cauda epididymal sperm were incubated without recombinant proteins, smeared on slides and incubated with anti-rec-BSPH1 antibodies at dilution of 1:100. For the two controls, cauda epididymal sperm were incubated with 15 µg rec-BSPH1 or 7 µg Trx-His-S and smeared on slide and incubated with IgG or His-probe antibodies respectively at dilution of 1:100. All slides were then treated with goat anti-mouse/anti-rabbit FITC-conjugated IgG and counterstained with Hoechst dye to localize nuclei (left panel).

Fig. 7. *Binding patterns of rec-BSPH1 on caudal sperm.* Sperm collected from mouse epididymides were incubated with 15 µg of rec-BSPH1 in WH media alone, in WH media supplemented with BSA or in WH media containing BSA and calcium ionophore A23187. Sperm were then fixed with 4% (w/v) paraformaldehyde and smeared on slides. Slides were incubated with anti-rec-BSPH1 antibodies and treated with FITC-conjugated goat-anti-rabbit IgG. Bar graphs show the percentage of sperm appearing under three binding patterns. 400 sperm were evaluated for each conditions and this experiment was repeated 4 times.

Fig 8. *Proposed model of sperm capacitation by BSPH1*. During epididymal maturation, BSPH1 protein binds to sperm surface to stabilize the membrane. Following ejaculation and the journey through the female genital tract, in the oviduct, sperm bound BSPH1 interacts with oviductal fluid components namely HDL. HDL would sequestrate BSPH1 along with lipids to destabilize the sperm membrane (capacitation).

FIGURE 1



E

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  1 MAQPLDFLLV SICLFHSLFS FQVEDYYAPT IESLIRNPET EDGACVFPFL
 51 YRSEIFYDCV NFNLKHKWCS LNKTYQGYWK YCALSDYAPC AFPFWYRHMI
101 YWDCTEDGEV FGKKWCSLTP NYNKDQVWKY CIE
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FIGURE 2

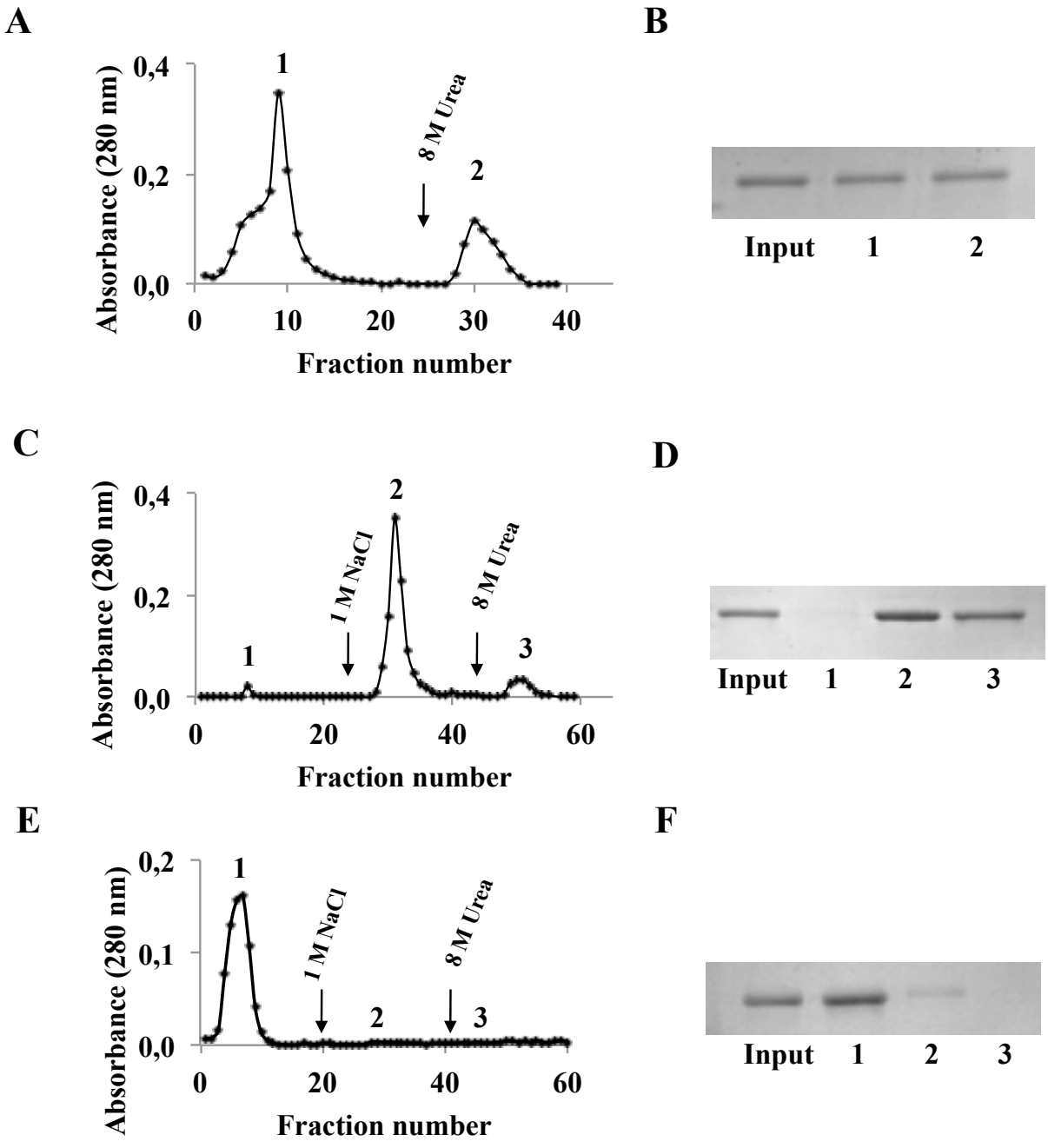

A
B
C
D
E
F
Absorbance (280 nm)
Fraction number
8 M Urea
1 M NaCl
Input
1
2
3

FIGURE 3

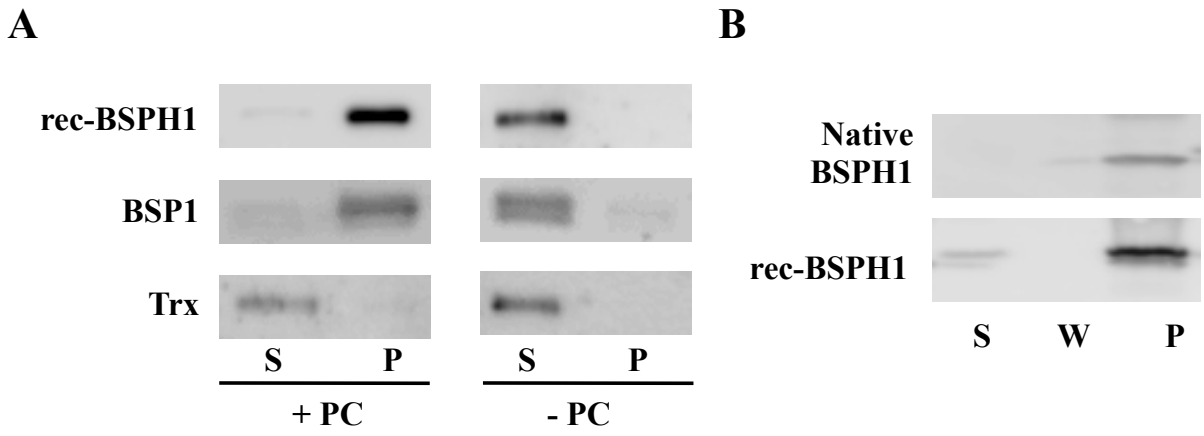
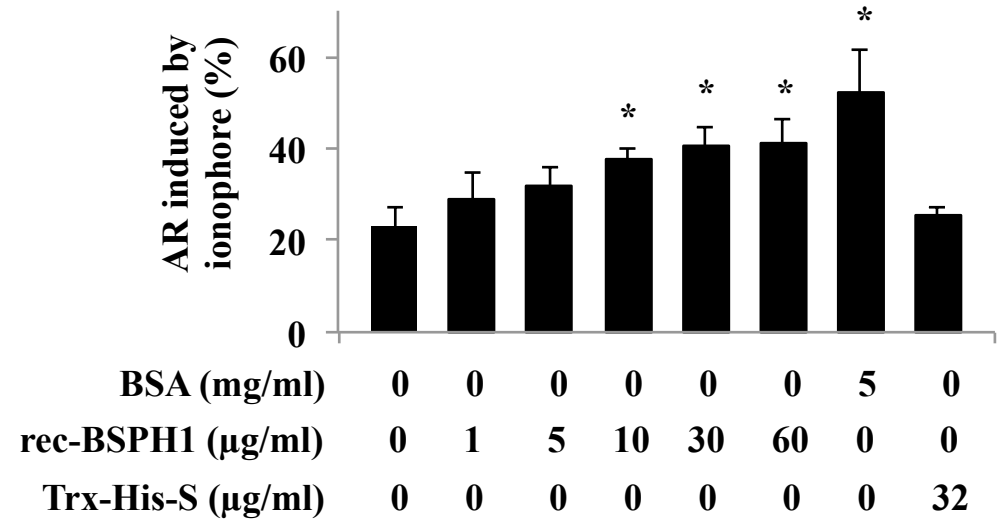


FIGURE 4

A


AR induced by ionophore (%)
60
40
20
0
*
*
*
*
BSA (mg/ml) 0 0 0 0 0 0 5 0
rec-BSPH1 (µg/ml) 0 1 5 10 30 60 0 0
Trx-His-S (µg/ml) 0 0 0 0 0 0 0 32

B

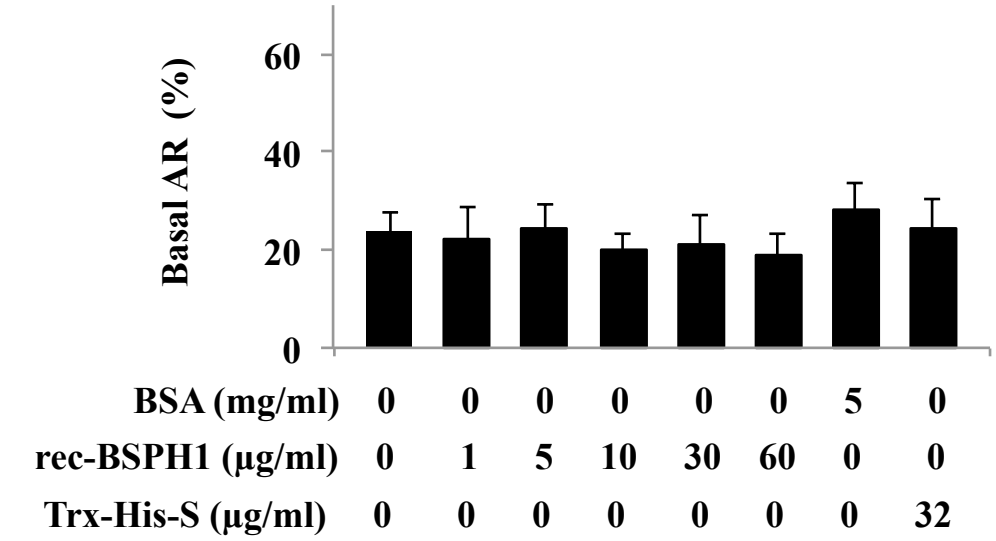

Basal AR (%)
60
40
20
0
BSA (mg/ml) 0 0 0 0 0 0 5 0
rec-BSPH1 (µg/ml) 0 1 5 10 30 60 0 0
Trx-His-S (µg/ml) 0 0 0 0 0 0 0 32

FIGURE 5

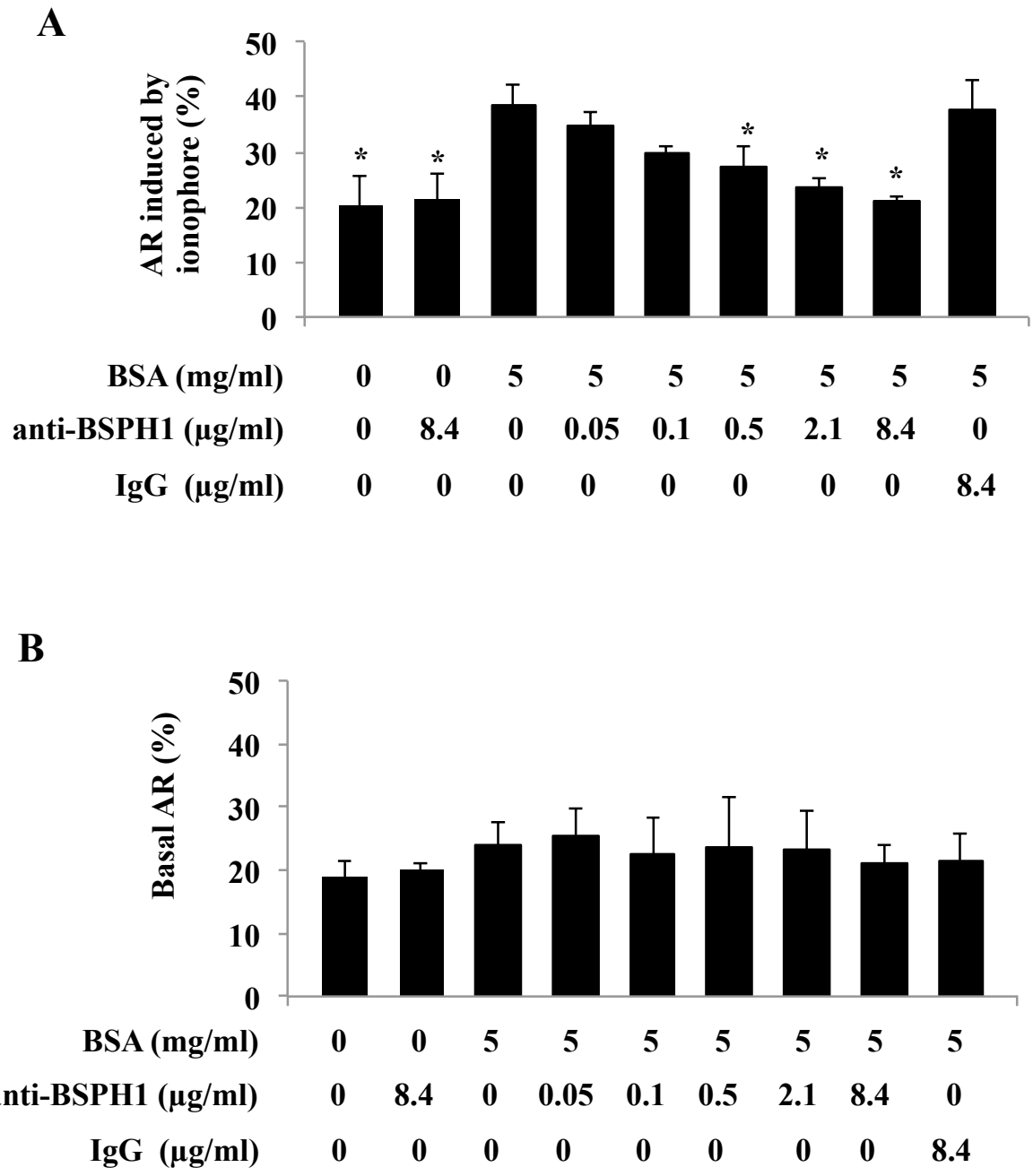

A
AR induced by ionophore (%)
50
40
30
20
10
0
*
*
*
*
*
BSA (mg/ml) 0 0 5 5 5 5 5 5 5
anti-BSPH1 (µg/ml) 0 8.4 0 0.05 0.1 0.5 2.1 8.4 0
IgG (µg/ml) 0 0 0 0 0 0 0 0 8.4
B
Basal AR (%)
50
40
30
20
10
0
BSA (mg/ml) 0 0 5 5 5 5 5 5 5
anti-BSPH1 (µg/ml) 0 8.4 0 0.05 0.1 0.5 2.1 8.4 0
IgG (µg/ml) 0 0 0 0 0 0 0 0 8.4

FIGURE 6

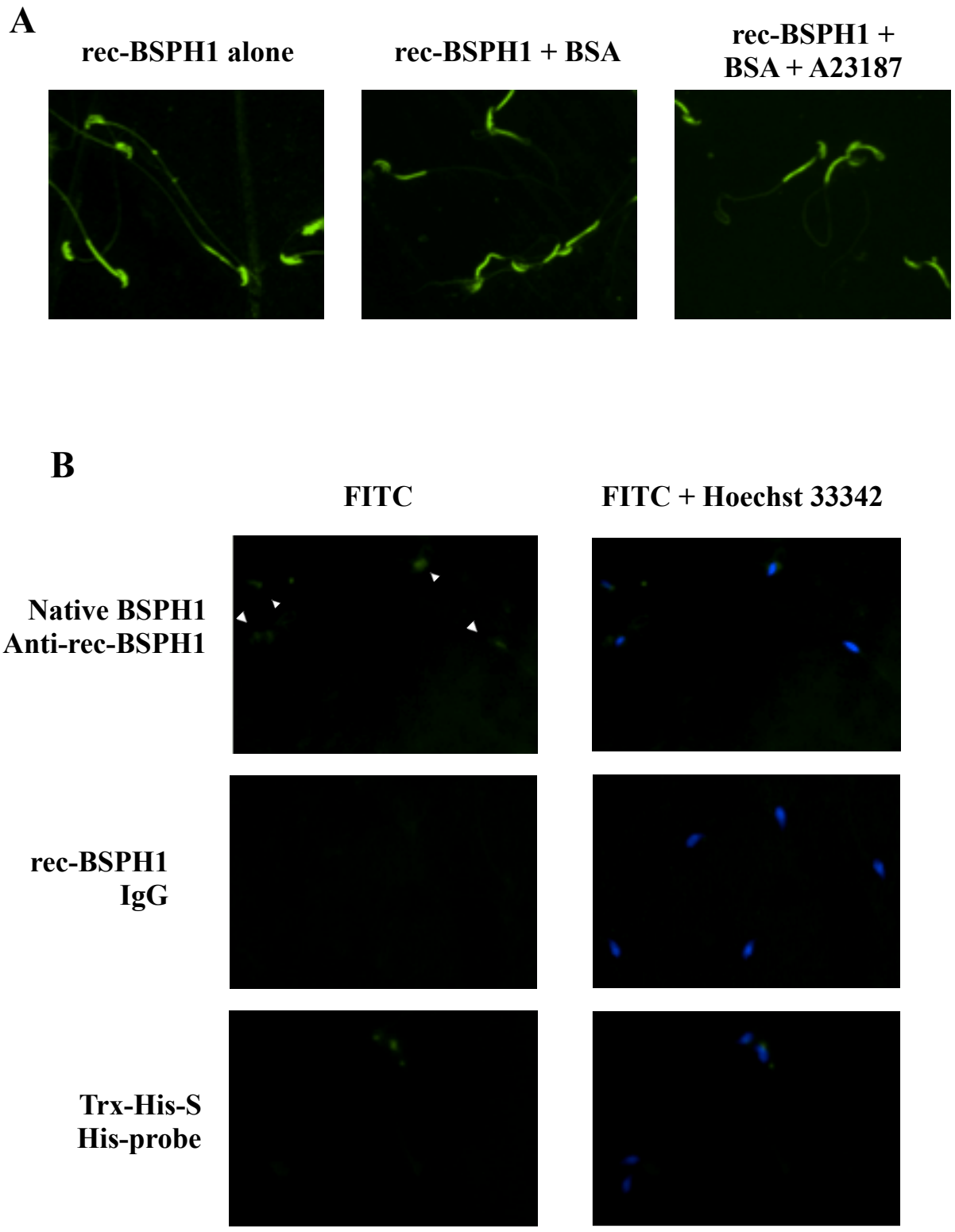

A
rec-BSPH1 alone
rec-BSPH1 + BSA
rec-BSPH1 + BSA + A23187
B
FITC
FITC + Hoechst 33342
Native BSPH1
Anti-rec-BSPH1
rec-BSPH1
IgG
Trx-His-S
His-probe

FIGURE 7

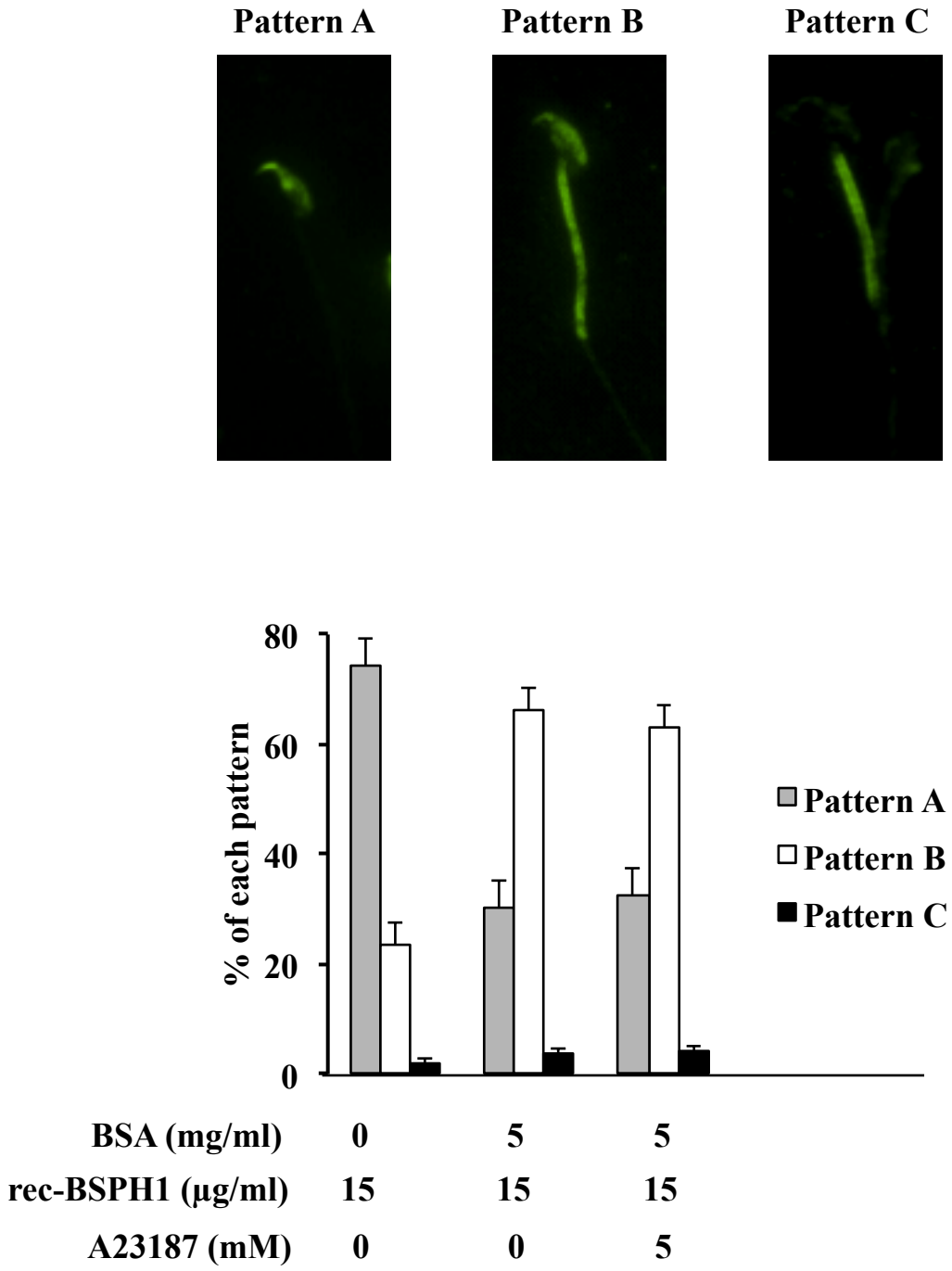
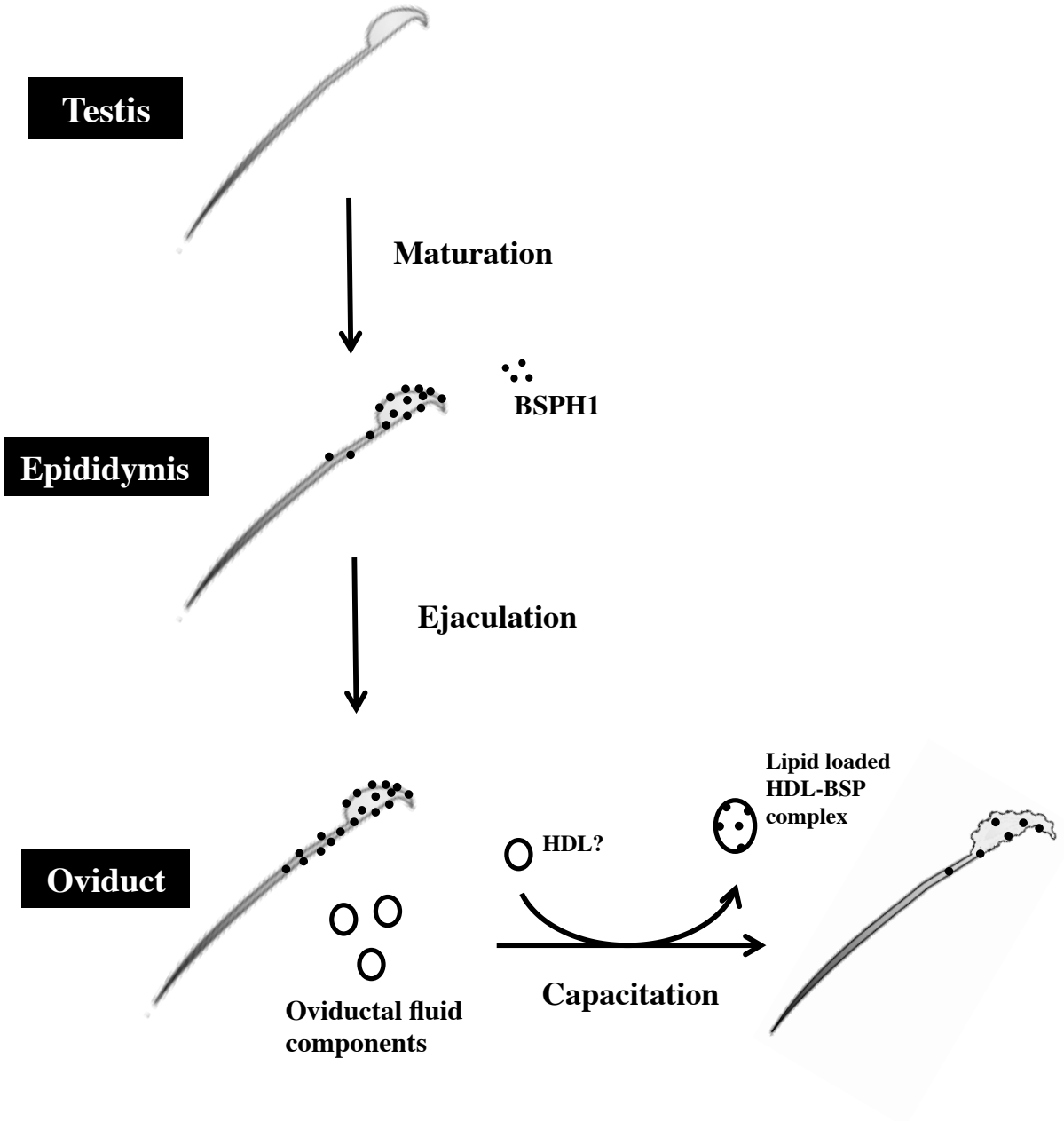

Pattern A
Pattern B
Pattern C
80
60
40
20
0
% of each pattern
Pattern A
Pattern B
Pattern C
BSA (mg/ml) 0 5 5
rec-BSPH1 (µg/ml) 15 15 15
A23187 (mM) 0 0 5

FIGURE 8


Testis
Maturation
BSPH1
Epididymis
Ejaculation
Oviduct
Lipid loaded
HDL-BSP
complex
HDL?
Oviductal fluid
components
Capacitation

Article 2

Implication of the human Binder of SPerm Homolog 1 (BSPH1) protein in capacitation.

Geneviève Plante, Isabelle Thérien, Catherine Lachance, Pierre Leclerc, Jinjiang Fan et Puttaswamy Manjunath. Publié dans *Molecular Human Reproduction* (2014) **20**(5), 409-21.

Résumé

Les protéines Binder of SPerm (BSP) sont une famille de protéines exprimées exclusivement dans le tractus génital mâle (vésicules séminales ou épididymes) de plusieurs mammifères. Le rôle principal de ces protéines est de promouvoir la capacitation, une étape de maturation des spermatozoïdes essentielle pour la fécondation d'un ovocyte. Nos études récentes ont démontré que, chez l'humain, la protéine Binder of SPerm Homolog 1 (BSPH1) est exprimée uniquement dans les tissus épididymaires. Le but de la présente étude était de caractériser BSPH1 et de tester son effet sur différentes fonctions spermatiques. Pour ce faire, une protéine recombinante BSPH1 (rec-BSPH1) a été produite, purifiée et repliée. Les résultats obtenus démontrent que rec-BSPH1 partage plusieurs caractéristiques avec les autres membres de la superfamille des BSP incluant la liaison à la gélatine et l'héparine et leur habileté à induire la capacitation des spermatozoïdes. Rec-BSPH1 n'a eu aucun effet sur la réaction acrosomique ou sur la motilité des spermatozoïdes. Des expériences de localisation ont démontré que la protéine BSPH1 native se lie au niveau du segment équatorial, du segment post-acrosomique et au niveau du cou des spermatozoïdes humains éjaculés. Rec-BSPH1 a montré un patron de liaison similaire à celui de la protéine native. Ces résultats démontrent que la protéine épididymaire BSPH1 partage plusieurs caractéristiques biochimiques et fonctionnelles avec les protéines BSP sécrétées au niveau des vésicules séminales des ongulés et se comporte de manière similaire à la protéine BSPH1 de souris.

Contribution

Pour cet article, j'ai contribué au design expérimental, à l'acquisition des données et à l'analyse des données pour l'expression et la purification de la protéine recombinante, les tests de liaison de ces protéines, l'immuno-localisation de BSPH1 sur les spermatozoïdes humains et pour

une partie des tests de motilité. J'ai également effectué les alignements de séquences. J'ai écrit l'article et effectué les corrections proposées lors de l'évaluation par les pairs.

Implication of the Human Binder of SPerm Homolog 1 (BSPH1) Protein in Capacitation.

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Short title: Roles of human rec-BSPH1 in sperm functions.

Key words: Binder of SPerm (BSP) proteins / epididymal protein / fibronectin type II (Fn2) domains / human sperm capacitation / recombinant protein

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 1 (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 (BSP) proteins / epididymal protein / fibronectin type II (Fn2) 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 approximately 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 (Bergeron *et al.,* 2005, Boisvert *et al.,* 2004, Calvete *et al.,* 1995a, Calvete *et al.,* 1997, Harshan *et al.,* 2009, Menard *et al.,* 2003, Villemure *et al.,* 2003). 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 (Lane *et al.,* 1999, Lusignan *et al.,* 2007, Therien *et al.,* 2005, Therien *et al.,* 1998).

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, de Lamirande *et al.,* 1997, Go and Wolf, 1983, Langlais and Roberts, 1985, Suarez, 1996, 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-related 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 BSPH1 and recombinant human 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 phosphatidylcholine (PC), egg yolk low-density lipoproteins (LDL) and human ejaculated sperm (Lefebvre *et al.*, 2009). Murine BSPH1 is orthologous to human BSPH1. They share about 56% identity and 78% similarity (Lefebvre *et al.*, 2007). Murine recombinant 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 $O.D._{600nm}$ reached 0.6-0.8. To induce the expression, 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 6000 × *g* for 10 min at 4°C.

Purification method was modified from Plante et al. (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 4X 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 20 000 × *g* 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 Ni^{2+} and equilibrated with 1X binding buffer containing 6 M urea at a flow rate of 24 ml/h. The column was washed with 5 bed volumes of 1X 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 M to 0 M) over 16 hours in 1X 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 $O.D._{280}$, similar quantities of all samples were precipitated with TCA (final concentration 15%) and analyzed by sodium dodecyl sulphate-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). 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 (Laemmli, 1970) in 15% polyacrylamide gels using the Mini-Protean 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 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 against a synthetic peptide corresponding to the last 15 C-terminal amino acids (SLTKNFNKDRIWKYCE) of the deduced sequence of BSPH1 (anti-16mer) or affinity-purified antibodies against $(His)_6$-tagged recombinant BSPH1 (anti-BSPH1) at a dilution of 1:1000 (Lefebvre *et al.*, 2009). Goat anti-mouse IgG (1:3000) or goat anti-rabbit IgG (1:10000) were used as secondary antibodies (Bio-

Rad). The bands were revealed using a chemiluminescence reagent (ECL kit, Perkin–Elmer, 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'Urfé, QC, Canada). The coupling of gelatin to agarose beads and of chondroitin sulphate B (CSB) (Sigma-Aldrich) to Affi-gel 15 were performed as previously described (Manjunath *et al.,* 1987, Plante *et al.*, 2012, Therien *et al.*, 2005). 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 approximately 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 BSP1 (PDB accession number 1H8P) available from the Brookhaven Protein Database (PDB) (Guex and Peitsch, 1997, Schwede *et al.,* 2003). Molecular docking analyses were performed using AutoDock-vina (Trott and Olson, 2010). The 3D coordinates for heparin heptasaccharide and CSB were retrieved from the ternary complex of antithrombin-anhydrothrombin-heparin (1SR5) and from the crystal structures of a cathepsin K:chondroitin sulfate complex (3C9E), respectively (http://www.rcsb.org) (Dementiev *et al.,* 2004, Li *et al.,* 2008).

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. (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 (BWW; 10 mM HEPES, 94.6 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.1 mM $NaHCO_3$, 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 Analyser (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% CO_2 in modified BWW medium alone or in the presence of different concentrations of rec-BSPH1 (0-60 µg/ml) or without protein, or with 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 PBS, centrifuged at $500 \times g$ 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 *Pisum sativum* 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 500 × *g* for 5 minutes. 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 hour at room temperature, washed and incubated with a GAM-HRP for 1 hour. 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 pre-warmed 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 (x10) (Nikon Canada Inc. Instruments, Mississauga, Ontario, Canada) connected to the SCA system. All motility parameters were evaluated for at least 8 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 greater than 75 μm/s, linearity less than 40**%** and amplitude of lateral head displacement greater than 3.5 (Boue *et al.,* 1994, Ferlin *et al.,* 2012).

Immunofluorescence

Sperm were incubated at 20 × 10^6 cells/ml for 1 h and 4 h (5% CO_2, 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 3 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 minutes at room temperature and washed another 3 times. Sperm were finally 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 minutes, washed 3 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-16mer (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 fluorescein isothiocyanate (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 ± S.E.M for acrosome reaction 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 *E. 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 Figure 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 to 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 further 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 (GAGs)

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 Figure 2A and B, when rec-BSPH1 was passed on a gelatin-agarose affinity chromatography column, about 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 approximately 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 -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 -4.5 kcal/mol (Fig. 3C). A bigger difference was observed for the binding of CSB as the GAG

appeared to bind in the front side groove between the Fn2 domains in BSPH1 with an affinity of -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 -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 h and 4 h incubation in BWW media at 37°C under 5% CO_2 (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 h 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.

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 ± 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 ± 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 ± 2 % when 60 µg/ml of protein was added. A significant increase

in the level of AR was reached using a minimum of 10 μg/ml of recombinant protein. The addition of 32 μg/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 μg/ml rec-BSPH1. However, the addition of 16 μg/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 h 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 ± 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 ± 3 % and 76 ± 2 % respectively). At different conditions tested total motility did not change significantly.

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

Finally, at the beginning of the incubation, the level of hyperactivation was 2.9 ± 0.4 % (Fig. 7C). After 2 h and 4 h incubation, levels of hyperactivation without any added proteins reached 6.1 ± 1.7 % and 4.6 ± 0.9 % respectively. The addition of different rec-BSPH1 concentrations, 3 mg/ml BSA or of 32 μg/ml of Trx-His-S did not cause any significant increase of the level of hyperactivation when compared to sperm incubated in BWW medium alone. Therefore, addition of rec-BSPH1, BSA or Trx-His-S did not affect any of the three motility parameters tested after 2 h 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 (Bessette *et al.,* 1999, Lefebvre *et al.*, 2009, Peisley and Gooley, 2007, Plante *et al.*, 2012, Prinz *et al.,* 1997).

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 tetramers, has been observed for native BSPH1 (Kumar *et al.,* 2008) as well as in BSP proteins of other species (Bergeron *et al.*, 2005, Calvete *et al.*, 1995a, Calvete *et al.,* 1995b, Gasset *et al.,* 1997, Manjunath and Sairam, 1987). 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.,* 1999, Calvete *et al.*, 1995a, Calvete *et al.*, 1995b).

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 (HDL), LDL, choline phospholipids and gelatin (Bergeron *et al.,* 2004, Bergeron *et al.*, 2005, Boisvert *et al.*, 2004, Calvete *et al.*, 1995a, Calvete *et al.*, 1997, Chandonnet *et al.,* 1990, Desnoyers and Manjunath, 1992, Lane *et al.*, 1999, Manjunath *et al.,* 2002, Manjunath *et al.*, 1987, Menard *et al.*, 2003, Sanz *et al.,* 1993, Therien *et al.*, 2005, Therien *et al.*, 1998, Villemure *et al.*, 2003). 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 (Hileman *et al.,* 1998, Manjunath *et al.*, 1987, Salek-Ardakani *et al.,* 2000). Some studies have also identified essential amino acid(s) responsible for the binding to different ligands (Calvete *et al.*, 1999, Collier *et al.,* 1992, 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 recombinant 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 (Jois and Manjunath, 2010, Wah *et al.*, 2002). 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.,* 2013). 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. 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 to 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% (~50 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 recombinant 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 (Lusignan *et al.*, 2007, Plante *et al.*, 2012, Therien *et al.,* 1995). 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 which can induce sperm capacitation. HDL as well as free BSP (unbound to sperm) appear to act as acceptors to induce the lipid efflux (Manjunath *et al.,* 2007, Therien *et al.*, 1998). 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 µg/ml of recombinant proteins cause significant increase in sperm capacitation (Plante *et al.*, 2012).

It has been shown that sphyngomyelin (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 (unbound) BSP to act as acceptors and induce lipid efflux (Manjunath *et al.*, 2007, Therien *et al.*, 1995, Therien *et al.,* 2001, Therien *et al.*, 1998). In a similar manner, in this study, when low concentrations are used (lower than 10 µg/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 sphyngomyelin, 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 to 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.

AUTHORS' ROLES

G.P. contributed to the design, acquisition of data and analysis of the experiments related to Figures 1, 2, 4, 7 and 8. I.T. contributed to the design, acquisition of data and analysis of the experiments related to Figures 4, 5 and 7. C.L. contributed to the design, acquisition of data and analysis of the experiments related to Figure 6. J.F. performed all bio-informatics analyses related to Figure 3. P.L contributed to the analysis and interpretation of the data of the experiments related to Figure 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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FIGURE LEGENDS

Fig. 1. *Expression and purification of rec-BSPH1*. (A) IMAC profile of the stepwise elution of rec-BSPH1 protein. (B) Analysis of the column fractions by 15% SDS-PAGE stained with Coomassie Blue R-250. I, input; P, cell extract pellet; FT, flow through; NA, nonadsorbed; W, wash; E1 (tubes 9-27), elution with 150 mM imidazole; E2 (tubes 28-48), elution with 200 mM imidazole and E3 (tubes 62-76), elution with 400 mM imidazole. Approximately, 15 μg proteins were loaded in each lane except lanes E1-3 where 7 μg proteins were loaded. The pellet (P) of the cell lysate, was resuspended in sample buffer and 1/100 of the fraction was loaded in lane P. (C) Western blot with anti-BSPH1 antibodies, under non-reducing conditions, of rec-BSPH1 purified without on-column refolding (lane 1) or purified with on-column refolding (lane 2; fraction E2). (D) LC-MS/MS analysis of the 32 kDa band in pooled E2-E3 fractions. Bold letters represent residues identified by LC-MS/MS.

Fig. 2. *Binding to gelatin and GAGs.* Affinity chromatography profiles and SDS-PAGE patterns of column fractions of rec-BSPH1 on gelatin-agarose (A, B), heparin-sepharose (C, D) and CSB-agarose (E, F). 500 μg of proteins were loaded on each column. The columns were then washed with 25 mM TB, and eluted with TB containing 1 M NaCl (GAGs columns) and 8 M urea and 100 mM choline chloride (gelatin column). These results are representative of at least three separate experiments.

Fig. 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 human BSPH1 (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.

Fig. 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 μg/ml

rec-BSPH1 or in the presence of 16/ μg/ml Trx-His-S for 1 h 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 × 630. (DIC: Differential Interference Contrast)

Fig. 5. *Effect of rec-BSPH1 on human sperm capacitation*. Capacitation was assessed by the ability of sperm to undergo AR induced by A23187 ionophore. Ejaculated sperm were separated on percoll gradient, incubated with different concentration of rec-BSPH1 for 4 h followed by an incubation in the presence (closed circles) or the absence (open circles) of A23187 ionophore for 30 min. Sperm were smeared and analyzed using PSA-FITC staining. Data are presented as the mean ± S.E.M of six independent experiments. Differences compared to control (sperm alone) were analyzed by one-way ANOVA followed by Bonferroni post hoc test. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

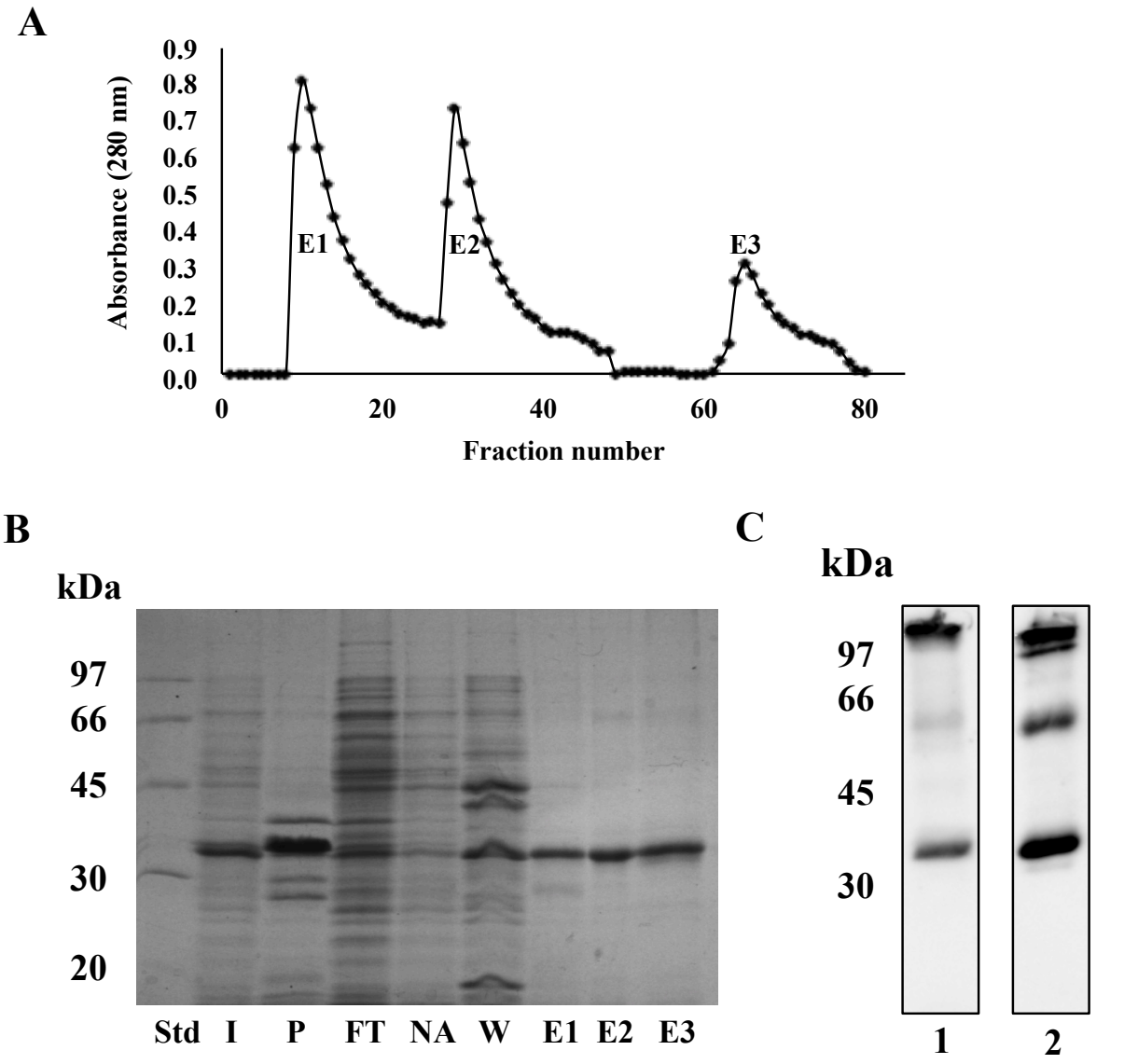
Fig. 6. *Effect of rec-BSPH1 on tyrosine phosphorylation.* Ejaculated sperm were separated on percoll gradient, incubated in the presence of different concentrations of rec-BSPH1 or Trx-His-S for 4 h. (A) Pattern of protein-tyrosine phosphorylation. Proteins were separated by SDS-PAGE, transferred on a PVDF membrane and probed with anti-phosphotyrosine antibody. Each membrane was re-probed with anti-alpha-tubulin antibody as proteins loading control. (B) Normalized signal of the phosphotyrosine content of p81 and of p105 to the α-tubulin content. Signal was scanned from each band and p105/tubulin and p81/tubulin ratios were established. Data are presented as the mean ± S.E.M of five independent experiments.

Fig. 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 ±

S.E.M of six independent experiments. Differences compared to control (sperm alone) were analyzed by one-way ANOVA followed by Bonferroni post hoc test.

Fig. 8. *Amino acid sequence alignments of BSP1, BSP3, human BSPH1 (hBSPH1) and murine BSPH1 (mBSPH1)*. The Fn2 domains are underlined. Asterisks (purple) indicate aromatic residues that bind phosphorylcholine and arrows (green), gelatin-binding residues.

FIGURE 1



D

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  1 IFPVILNELS STVETITHFP EVTDGECVFP FHYKNGTYYD CIKSKARHKW
 51 CSLNKTYEGY WKFCSAEDFA NCVFPFWYRR LIYWECTDDG EAFGKKWCSL
101 TKNFNKDRIW KYCE
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FIGURE 2

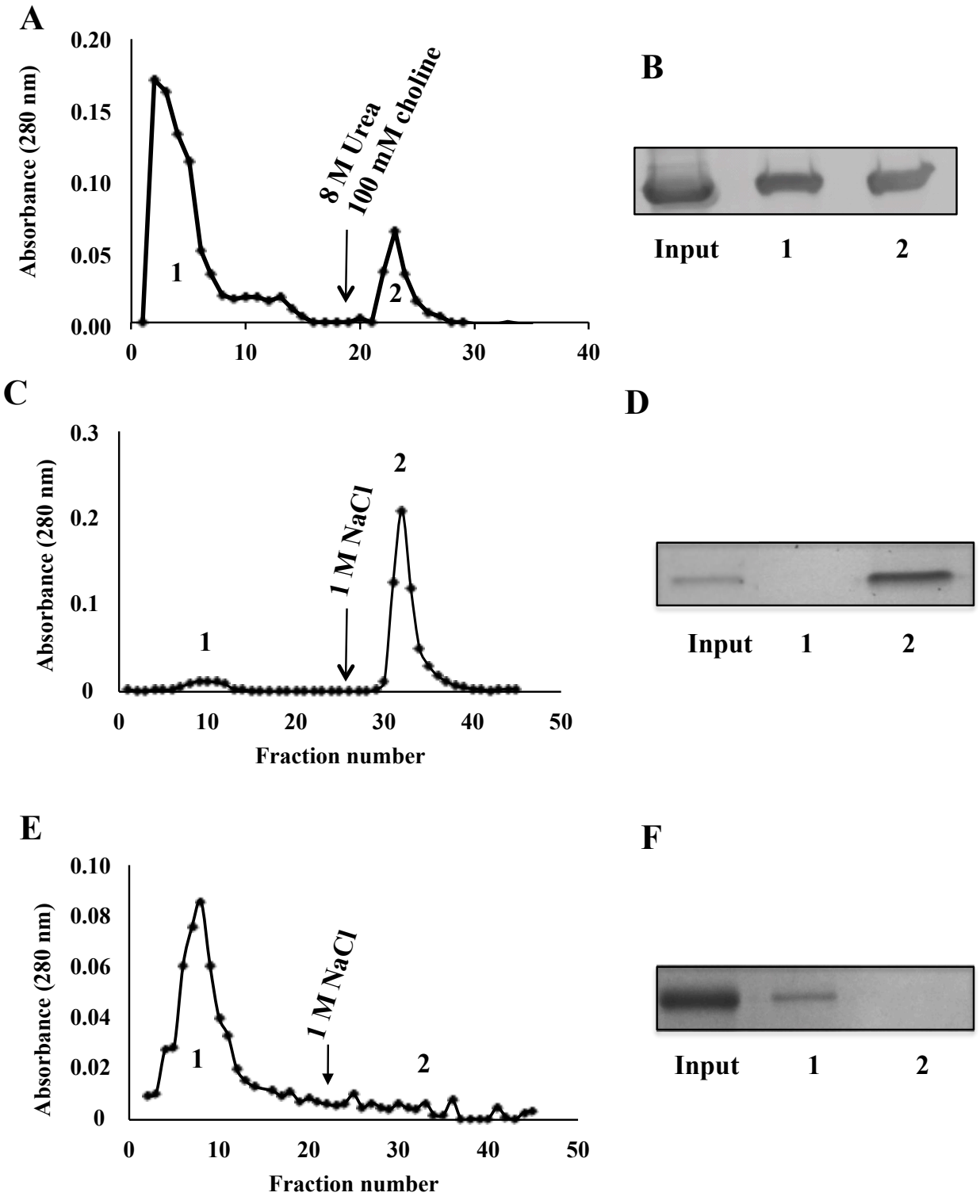

A
Absorbance (280 nm)
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
8 M Urea
100 mM choline
1
2
B
Input
1
2
C
Absorbance (280 nm)
0.3
0.2
0.1
0
0
10
20
30
40
50
Fraction number
1 M NaCl
1
2
D
Input
1
2
E
Absorbance (280 nm)
0.10
0.08
0.06
0.04
0.02
0
0
10
20
30
40
50
Fraction number
1 M NaCl
1
2
F
Input
1
2

FIGURE 3

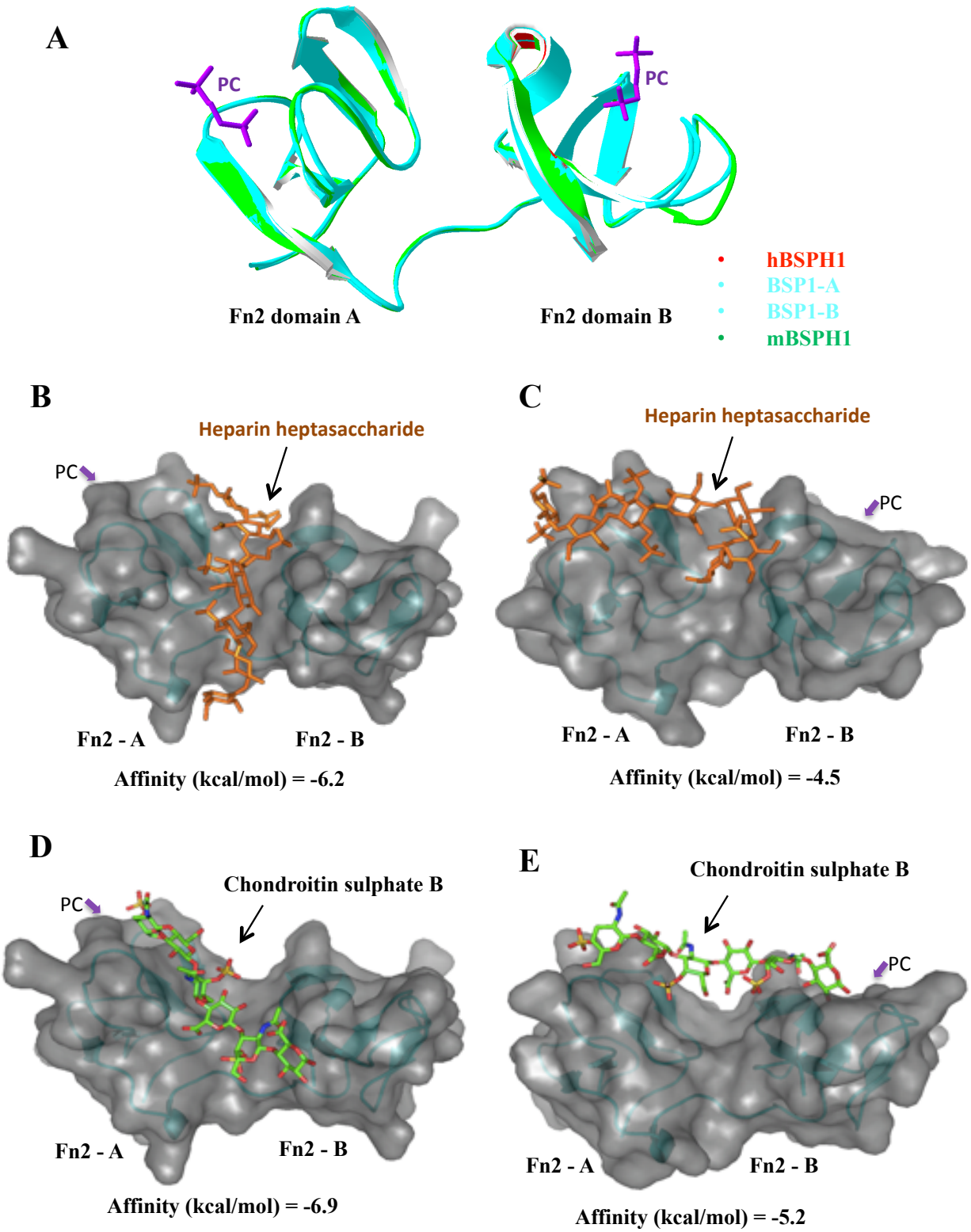

A
PC
PC
hBSPH1
BSP1-A
BSP1-B
mBSPH1
Fn2 domain A
Fn2 domain B
B
Heparin heptasaccharide
PC
Fn2 - A
Fn2 - B
Affinity (kcal/mol) = -6.2
C
Heparin heptasaccharide
PC
Fn2 - A
Fn2 - B
Affinity (kcal/mol) = -4.5
D
Chondroitin sulphate B
PC
Fn2 - A
Fn2 - B
Affinity (kcal/mol) = -6.9
E
Chondroitin sulphate B
PC
Fn2 - A
Fn2 - B
Affinity (kcal/mol) = -5.2

FIGURE 4

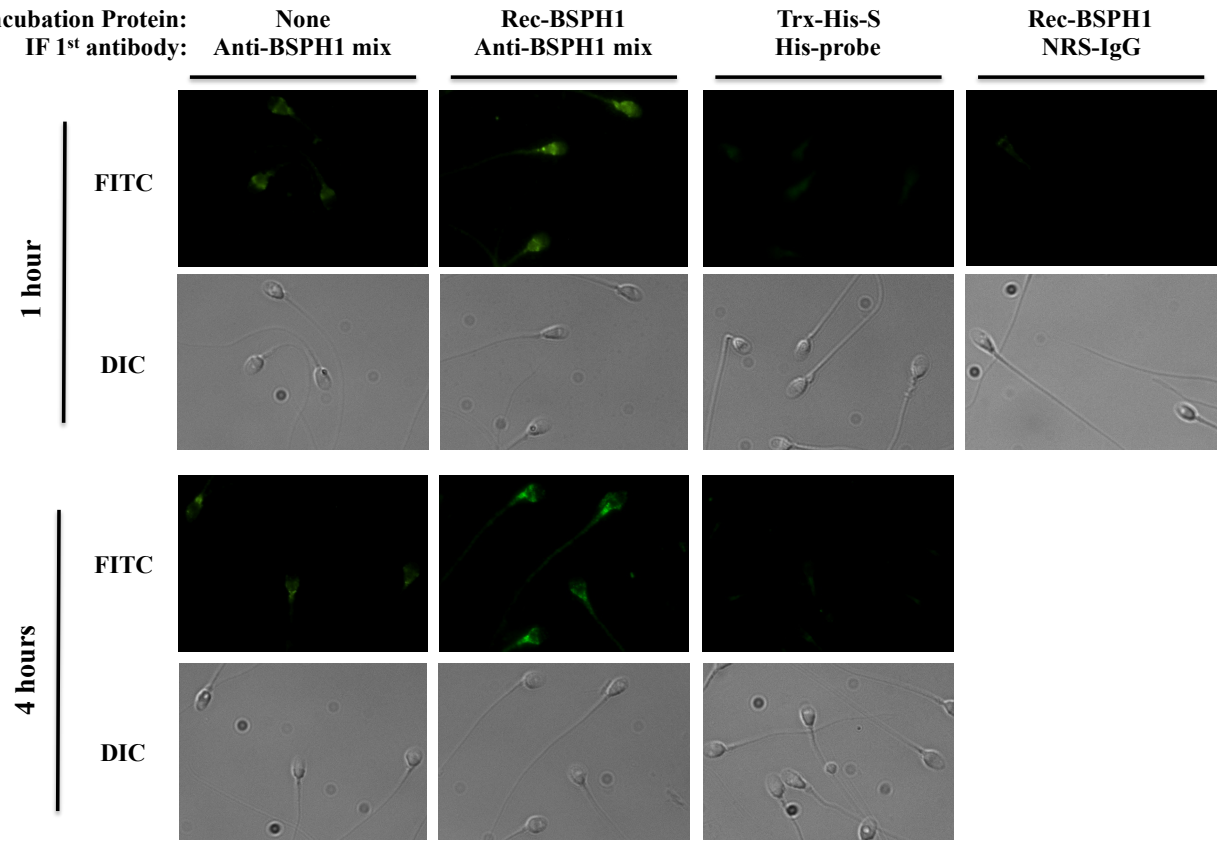

Incubation Protein:
IF 1st antibody:
None
Anti-BSPH1 mix
Rec-BSPH1
Anti-BSPH1 mix
Trx-His-S
His-probe
Rec-BSPH1
NRS-IgG
1 hour
FITC
DIC
4 hours
FITC
DIC

FIGURE 5

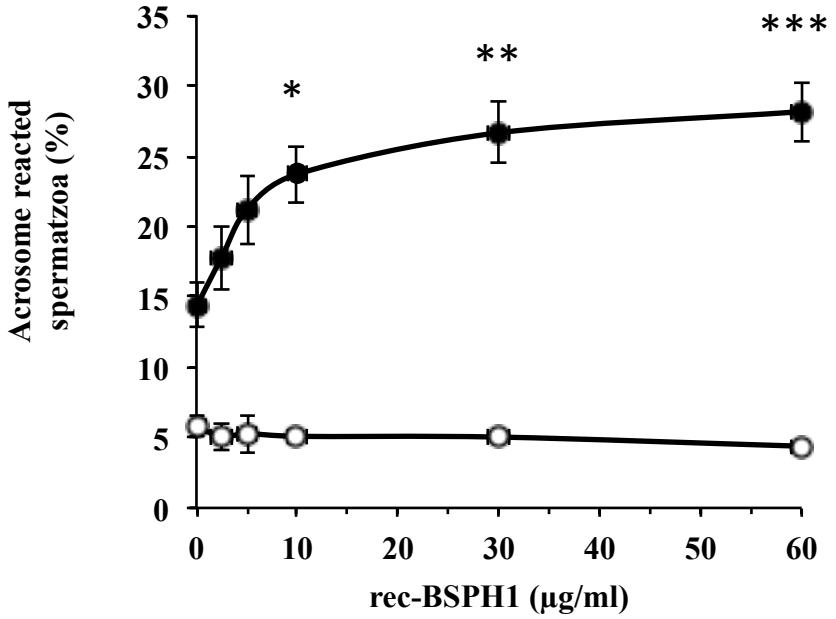

Acrosome reacted spermatzoa (%)
35
30
25
20
15
10
5
0
*
**
***
0
10
20
30
40
50
60
rec-BSPH1 (µg/ml)

FIGURE 6

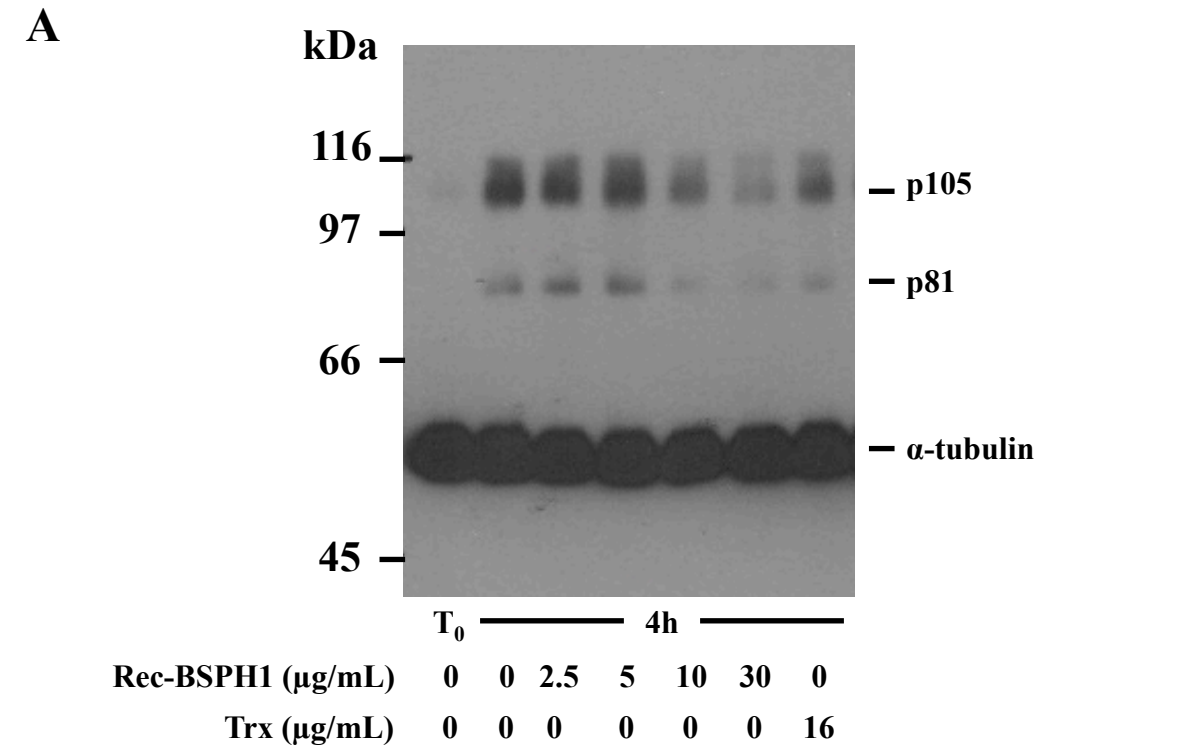

A
kDa
116
97
66
45
p105
p81
α-tubulin
T0
4h
Rec-BSPH1 (µg/mL) 0 0 2.5 5 10 30 0
Trx (µg/mL) 0 0 0 0 0 0 16
B
Arbitrary Units
15
10
5
0
p105
p81
T0
4h
Rec-BSPH1 (µg/mL) 0 0 2.5 5 10 30 0
Trx (µg/mL) 0 0 0 0 0 0 16

FIGURE 7

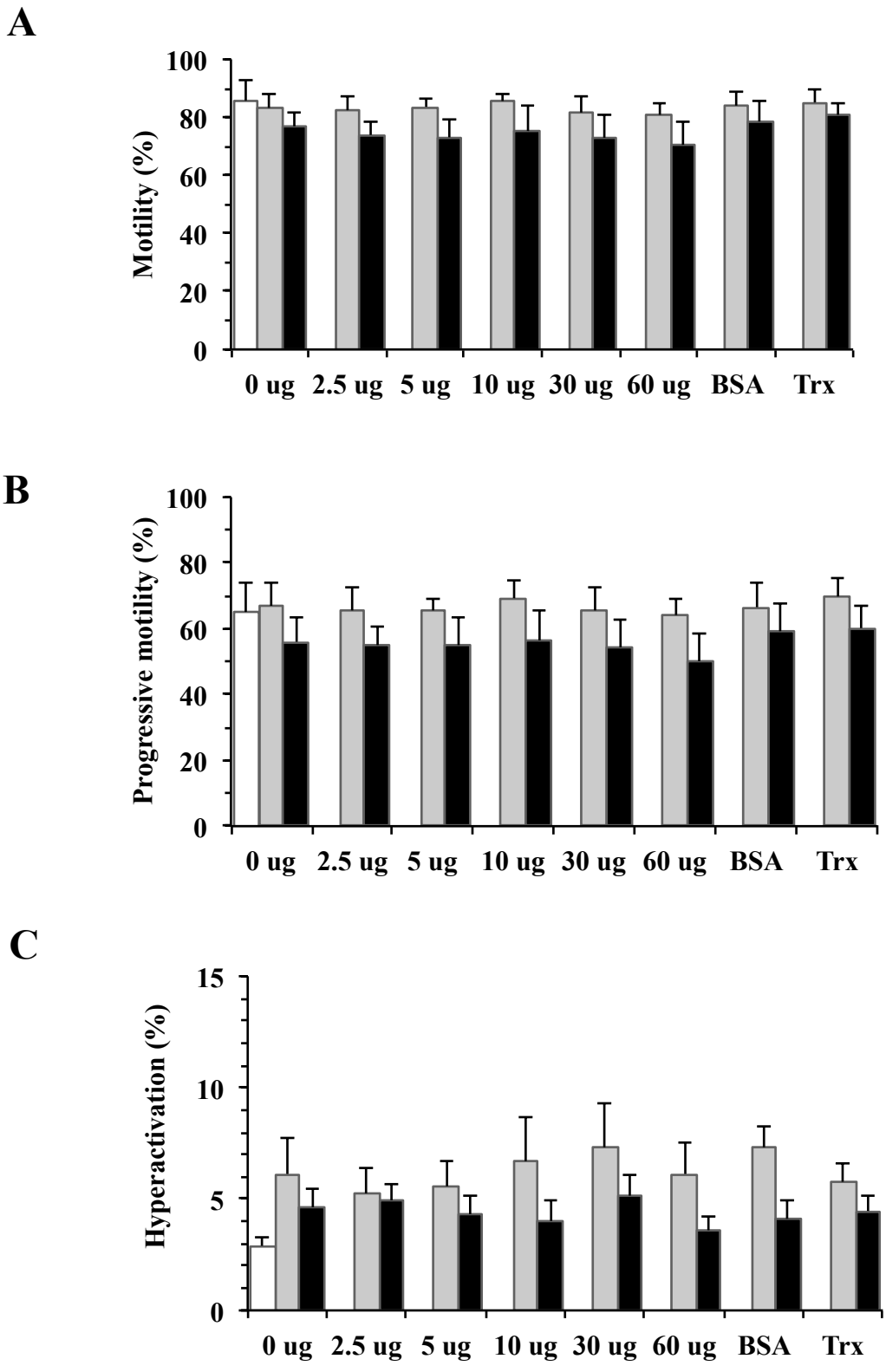
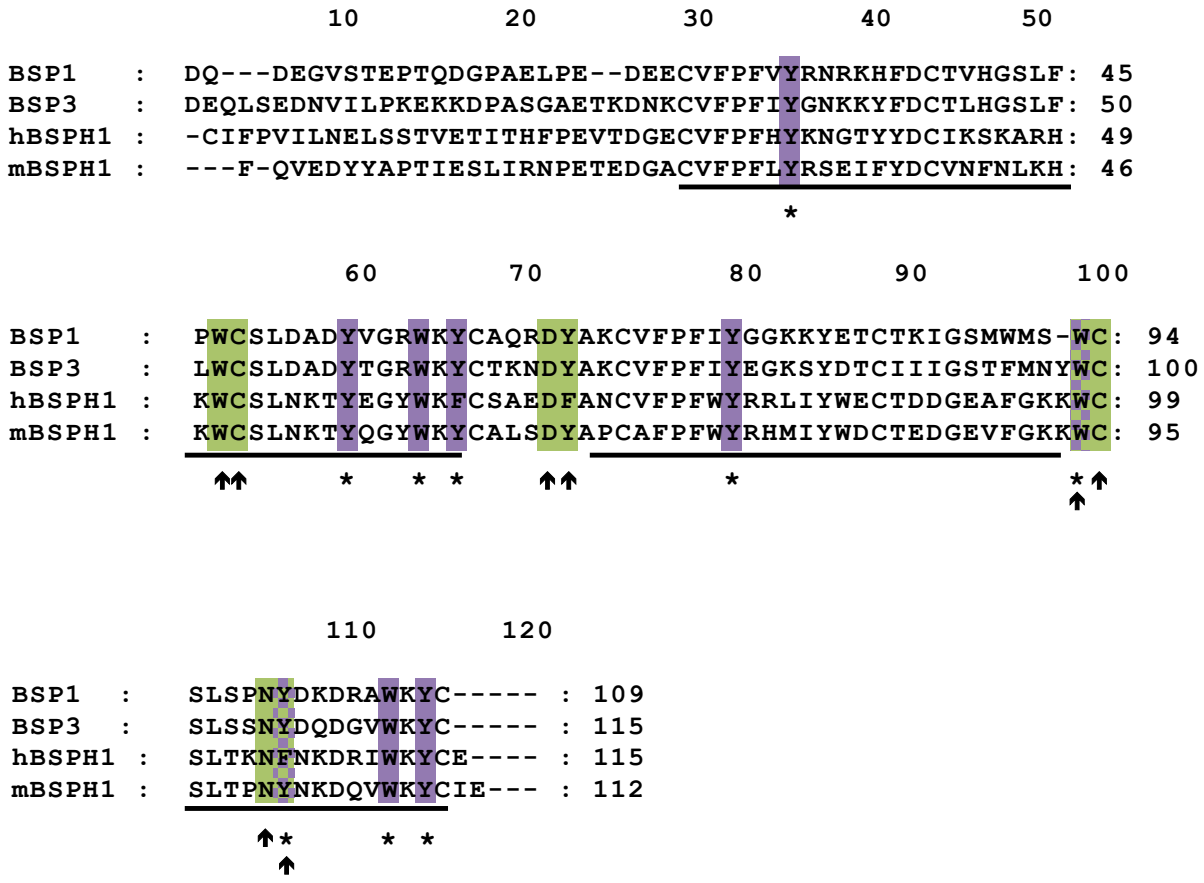

A
Motility (%)
100
80
60
40
20
0
0 ug
2.5 ug
5 ug
10 ug
30 ug
60 ug
BSA
Trx
B
Progressive motility (%)
100
80
60
40
20
0
0 ug
2.5 ug
5 ug
10 ug
30 ug
60 ug
BSA
Trx
C
Hyperactivation (%)
15
10
5
0
0 ug
2.5 ug
5 ug
10 ug
30 ug
60 ug
BSA
Trx

FIGURE 8


10 20 30 40 50
BSP1 : DQ---DEGVSTEPTQDGPAELPE--DEECVFPFVYRNRKHFDCTVHGSLF: 45
BSP3 : DEQLSEDNVILPKEKKDPASGAETKDNKCVFPFIYGNKKYFDCTLHGSLF: 50
hBSPH1 : -CIFPVILNELSSTVETITHFPEVTDGECVFPFHYKNGTYYDCIKSKARH: 49
mBSPH1 : ---F-QVEDYYAPTIESLIRNPETEDGACVFPFLYRSEIFYDCVNFNLKH: 46
60 70 80 90 100
BSP1 : PWCSLDADYVGRWKYCAQRDYAKCVFPFIYGGKKYETCTKIGSMWMS-WC: 94
BSP3 : LWCSLDADYTGRWKYCTKNDYAKCVFPFIYEGKSYDTCIIIGSTFMNYWC: 100
hBSPH1 : KWCSLNKTYEGYWKFCSAEDFANCVFPFWYRRLIYWECTDDGEAFGKKWC: 99
mBSPH1 : KWCSLNKTYQGYWKYCALSDYAPCAFPFWYRHMIYWDCTEDGEVFGKKWC: 95
110 120
BSP1 : SLSPNYDKDRAWKYC----- : 109
BSP3 : SLSSNYDQDGVWKYC----- : 115
hBSPH1 : SLTKNFNKDRIWKYCE---- : 115
mBSPH1 : SLTPNYNKDQVWKYCIE--- : 112

Article 3

Murine Binder of SPerm Homolog 2 (BSPH2): The Black Sheep of the BSP Superfamily.
Geneviève Plante, Jinjiang Fan et Puttaswamy Manjunath. Publié dans *Biology of Reproduction* (2014) **90**(1):20, 1-12.

Résumé

Les protéines de la famille Binder of SPerm (BSP), se lient aux phospholipides portant des groupements choline à la surface de la membrane des spermatozoïdes et promeuvent la capacitation. Cette étude porte sur la caractérisation des propriétés biochimiques et fonctionnelles de la protéine Binder of SPerm Homolog 2 (BSPH2) de souris. Une protéine recombinante (rec-BSPH2) a été exprimée dans des cellules *E.coli* Rosetta-gami B(DE3)pLysS avec un vecteur pET32a. Cette protéine a été purifiée par chromatographie d'affinité sur ions métalliques immobilisés et repliée sur colonne à l'aide d'un gradient d'urée décroissant. Les résultats obtenus démontrent que la protéine rec-BSPH2 partage certaines propriétés de liaison avec les autres protéines de la famille BSP tel la liaison à la gélatine, à l'héparine et aux spermatozoïdes épididymaires. Rec-BSPH2 et la protéine recombinante BSPH1 de souris se sont avérés avoir des patrons d'immunofluorescence différents sur les spermatozoïdes non capacités vs capacités, indiquant un réarrangement de ces protéines sur la surface des spermatozoïdes pendant ou après la capacitation. Étonnement, rec-BSPH2 ne peut pas se lier aux liposomes de phosphatidylcholine ou promouvoir la capacitation des spermatozoïdes. Des résultats similaires n'ont jamais été observés chez les autres protéines de la famille des BSP. Les résultats de cette étude indiquent que les protéines de souris BSPH1 et BSPH2 n'ont peut-être pas des fonctions redondantes dans la fécondation comme c'est le cas pour les protéines BSP bovines.

Contribution

J'ai contribué à environ 95% du développement conceptuel de l'article et j'ai choisi les expériences et les analyses bio-informatiques à faire. J'ai réalisé toutes les expériences *in vitro* décrites dans cet article scientifique et j'ai effectué l'analyse et l'interprétation de tous les résultats obtenus. J'ai construit l'article à partir des résultats obtenus. Dr Fan a réalisé les analyses bio-informatiques. J'ai rédigé l'article à l'exception des descriptions bio-informatiques et ai effectué les changements suite à l'évaluation par les pairs.

ABSTRACT

Proteins of the Binder of SPerm superfamily are known to bind choline phospholipids on sperm membrane and promote sperm capacitation. The current study focuses on the biochemical and functional characterization of the murine Binder of SPerm Homolog 2 (BSPH2). A recombinant protein (rec-BSPH2) was expressed in *E.coli* Rosetta-gami B(DE3)pLysS cells using pET32a vector. It was purified by immobilized metal ion affinity chromatography and refolded on column using a decreasing urea gradient. Rec-BSPH2 was found to share some binding characteristics with other BSP proteins such as binding to gelatin, heparin, and epididymal sperm. Rec-BSPH2 as well as murine recombinant BSPH1 were found to have different immunofluorescence patterns when bound to uncapacitated vs capacitated sperm, indicating a rearrangement of these proteins on sperm surface during or following capacitation. Surprisingly, rec-BSPH2 was unable to bind phosphorylcholine liposomes or promote sperm capacitation. It is the first time that such results are reported for proteins of the BSP family. The results indicate that murine BSPH1 and BSPH2 might not have redundant functions, as is the case with bovine BSPs. This study could lead to a better understanding of the role of BSP proteins in sperm functions and the existence of redundant BSP proteins in the reproductive tract.

INTRODUCTION

Sperm leaving the testis are unable to fertilize an oocyte. Many subsequent maturation steps are necessary for the fertilization to occur. Transit through the epididymis is one of the essential steps. Changes that occur during epididymal transit allow sperm to become motile and acquire factors required for sperm to become capacitation-competent. Capacitation is another important sperm maturation step, which takes place in the female genital tract [1, 2]. Molecular basis of sperm capacitation are not well understood, but it is usually associated with changes in the lipid composition of the sperm plasma membrane, increase in intracellular pH, increased permeability to ions such as calcium and increase in protein tyrosine phosphorylation [3-7]. Capacitation is also accompanied by a series of time-dependent phosphorylation events, activating many different signaling pathways including the protein kinase A (PKA) pathway, the protein kinase C (PKC) pathway, the extracellular signal-regulated kinase (ERK) pathway and the phosphatidyl-inositol-3-kinase (PI3K)/Akt pathway [7-13].

Genes coding for proteins from the Binder of SPerm (BSP; previously called Bovine Seminal Plasma proteins [14]) superfamily are expressed in the epididymis of mice (*Bsph1* and *Bsph2*; accession numbers DQ227498 and DQ227429) and human (*BSPH1*; accession number DQ227497) [15]. In mice, *Bsph1* is expressed mostly in the proximal region of the epididymis, whereas *Bsph2* can also be detected in more distal regions [16, 17].

BSP proteins were first identified in the bovine seminal plasma where they represent approximately 60% of the protein content [18-20]. It has been demonstrated that the three bovine BSP proteins (BSP1, BSP3 and BSP5, previously called PDC-109 or BSP-A1/A2, BSP-A3 and BSP-30K respectively [14]) have redundant functions. They bind to sperm via an interaction with choline phospholipids and promote capacitation induced by glycosaminoglycans (GAG) and high-density lipoproteins (HDL) [21-24]. Other functions attributed to bovine BSP proteins include binding to the oviductal epithelium and prolonging sperm motility and viability [25, 26].

In the bovine, BSP proteins are expressed by the seminal vesicles. It is also the case for BSP homologs found in other ungulates such as stallion, boar, goat, bison and ram [27-32]. All BSP proteins have a common structure. They possess a variable N-terminal domain followed by two finbronectin type-II (Fn2) domains arranged in tandem [14]. Human and mouse BSP proteins are slightly different as they possess a C-terminus tail [14, 17]. This family of proteins also shares biochemical and functional characteristics such as their ability to promote sperm capacitation and

their ability to bind to gelatin, GAGs, HDL, low-density lipoproteins and choline phospholipids [24, 30-34].

Not much is known about human and murine epididymal BSP proteins. Recently, recombinant human BSPH1 (manuscript in preparation) and recombinant murine BSPH1 (rec-BSPH1) [35] were produced and shown to share many biochemical properties with BSP proteins from ungulates. They were also able to promote sperm capacitation in vitro.

The aim of the current study was to determine if the two murine BSP proteins share binding characteristics and have redundant functions, as reported for bovine BSP proteins. Since it is not feasible to obtain BSP proteins from mice in sufficient quantities, a recombinant murine Binder of SPerm homolog 2 protein (rec-BSPH2) was expressed in *E. coli*, purified and used to perform functional assays.

MATERIALS AND METHODS

Cloning of cDNA sequence into the expression vector

For the expression of the N-terminal His-tagged BSPH2, murine epididymal cDNA was used as template for the Polymerase Chain Reaction (PCR) amplification of *Bsph2*. To clone the *Bsph2* coding sequence in a pET15b vector (Novagen, EMD Biosciences, La Jolla, CA, USA), the following oligonucleotide primers were used: Bsph2-F1 (*NdeI*) 5'-GCG CAT ATG GAA TTG ATC TCT CAT TTA CAT CCT CC-3' and Bsph2-R1 (*EcoRI*) 5'-CGA GAA TTC ATA CCT AAA AAT TGT TAG GAG AAC ATT GC-3'.

For the expression of BSPH2 fused to a thioredoxin-$(His)_6$-S-tag (Trx-His-S), the pET15b-Bsph2 vector was used as template. The following oligonucleotide primers were used to allow the sub-cloning in the pET32a expression vector (Novagen): Bsph2-F2 (*NcoI*) 5'-GAG CCA TGG AAT TGA TCT CTC ATT TAC ATC CTC-3' and Bsph2-R2 (*EcoRI*) 5'-GCA GAA TTC ATA CCT AAA AAT TGT TAG GAG AAC ATT GC-3'. The PCR was done using a *pfu* polymerase (Fermentas, Burlington, ON, Canada) under the following cycles: initial denaturation at 94°C for 3 min, 33 cycles of 94°C, 45 s; 60°C, 45 s; 72°C, 1 min and a final elongation step of 72°C for 7 min. *Taq* polymerase (GE Healthcare, Baie d'Urfé, QC, Canada) was added before the final elongation step to allow sub-cloning in a pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). The *Bsph2* coding sequence was then extracted from the pCR2.1 vector and the pET32a expression vector was linearized using the proper restriction enzymes (England BioLabs, Beverly, MA,

USA). Digested products were run on agarose gel, purified using Qiaex II gel extraction kit (Qiagen, Mississauga, ON, Canada) and ligated overnight using T_4 DNA ligase (Invitrogen). Ligation reactions were transformed into competent DH10β cells, and plasmid DNA was isolated using the QIAprep spin miniprep kit (Qiagen). Sequences were confirmed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Protein expression in E. coli

The positive plasmids were transformed in Rosetta-gami B(DE3)pLysS competent cells (Novagen) using standard methods. Transformed cells were then plated on Luria–Bertani (LB)-agar plates containing 100 μg/ml of ampicillin (Sigma–Aldrich, Oakville, ON, Canada) and incubated at 37°C overnight after which single colonies were inoculated in liquid LB medium containing the same concentration of ampicillin. For the protein expression, 250 ml of LB medium containing the same antibiotic were inoculated with 1/100 volume of overnight culture and bacteria were incubated at 37°C with shaking at 200 rpm until $O.D_{600nm}$ reached 0.6-0.8. To induce the expression, IPTG (Invitrogen) was added to the cell culture to a final concentration of 1 mM and the cells were transferred at 15°C, 200 rpm for 16 h. After induction, cells were harvested by centrifugation at 6000 × *g* for 10 min at 4°C.

Immobilized metal ion affinity chromatography (IMAC) and refolding

The purification method was modified from Plante et al. [35]. Cell pellets were resuspended in B-Per bacterial protein extraction reagent (Pierce, Rockford, IL, USA) as described by the manufacturer and subjected to sonication (ten cycles of 30 s on ice, with 1 min wait between bursts). One volume of 4X binding buffer (2 M NaCl, 80 mM Tris-HCl, 20 mM imidazole pH 7.4) was added to the cell lysate. Urea was added to a final concentration of 6 M and the volume was adjusted with water to four times the initial volume. The cell extract was finally centrifuged at 4°C for 30 min at 25 000 × *g* to separate the soluble and insoluble fractions. The soluble fraction was filtered through a 5 μm filter and loaded, at a flow rate of 24 ml/h, on a column (1 cm × 15 cm) containing 5 ml of His-Bind resin (Novagen) charged with Ni^{2+} and equilibrated with 1X binding buffer containing 6 M urea. The column was washed with 5 bed volumes of 1X binding buffer and 5 bed volumes of washing buffer (500 mM NaCl, 20 mM Tris-HCl, 80 mM

imidazole, 6 M urea, pH 7.4). The refolding of the bound proteins was done on-column, over 16 hours with a decreasing urea gradient (6 M to 0 M) in 1X 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 7.4 containing 70, 250 and 400 mM imidazole respectively). Based on optical density, similar quantities of all samples were precipitated with TCA (final concentration 15%) and analyzed by Sodium dodecyl sulphate-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) [36]. Trx-His-S control was prepared in the same way using Origami B (DE3)pLysS containing empty pET32a vectors and were purified by IMAC in the absence of urea.

Protein electrophoresis and western blotting

SDS–PAGE was performed according to the method of Laemmli [37] in 12 or 15% gels using the Mini-Protean 3 apparatus from Bio-Rad (Mississauga, ON, Canada). Gels were either stained with Coomassie Brilliant Blue R-250 (BioRad), or transferred electrophoretically to Immobilon-P 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 against a synthetic peptide corresponding to the last 15 C-terminal amino acids of the deduced sequence of BSPH1 (anti-15mer), affinity-purified antibodies against $(His)_6$-tagged recombinant BSPH1 (anti-BSPH1), or affinity-purified antibodies against bovine BSP1 (anti-BSP1) at dilutions of 1:1000 [24, 35]. 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 chemiluminescence reagent (Perkin–Elmer, 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'Urfé, QC, Canada). Coupling of gelatin and chondroitin sulphate B (CSB; Sigma-Aldrich) to Affi-gel 15 was performed as previously described [20, 24, 35]. For each experiment, 5 ml of resin was packed in a column (1 cm × 3.5 cm) and equilibrated with 50

mM Tris-HCl buffer, pH 7.4 (TB). 500 μg (gelatin and CSB) or 5 mg (heparin) of rec-BSPH2 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. The unbound material was washed from the column with TB and bound proteins were eluted with TB containing 1 M NaCl for heparin-sepharose and CSB-agarose affinity chromatography or with TB containing 8 M urea for the gelatin-agarose affinity chromatography. For quality control, alcohol precipitates of bovine seminal plasma proteins were run similarly on each column.

Binding to liposomes

Phosphatidylcholine (PC) liposomes were prepared as previously described [21]. Briefly, 10 mg of soy PC (L-α-phosphatidylcholine 95%; Avanti Polar Lipids inc., Alabaster, AL, USA) were evaporated under N_2 to form a thin film at the bottom of a glass test tube. Two ml of buffer A (10 mM Tris-HCl, 100 mM KCl, pH 7.5) was added to the tube and sonicated in a Branson ultrasonic water bath (model 3510) for approximately 20 min at room temperature. Small unilamellar liposomes were sedimented at 100 000 × *g* for 30 min at 25°C and resuspended in 1 ml of buffer B (10 mM Tris-HCl, 100 mM KCl, 2.5 mM $CaCl_2$, pH 7.5). Liposomes composed of PC, phosphatidylethanolamine (PE) and phosphatidylinositol (PI) or composed of PC and cholesterol (CHO) were prepared the same way using 7 mg of PC, 2 mg of PE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine > 99%; Avanti Polar Lipids inc.) and 1 mg of PI from pig liver (Serdany Research lab, London, ON, Canada) or 8.9 mg of PC and 1.1 mg of CHO from sheep's wool (Sigma-Aldrich). Rec-BSPH2 (20 µg) or bovine BSP1 (10 µg) were incubated with 300 µg of liposomes in 300 µl of buffer B for 40 min at room temperature. Liposomes were then sedimented at 100 000 × *g* for 45 min. An equal fraction of the supernatant and pellet (one third) were vortexed (1 min) with 1.1 ml of solvent (methanol/water/chloroform; 3:2:1, v/v). After centrifugation for 1 min at 10 000 × *g,* the upper phase was removed and vortexed (30 sec) with 400 µl of methanol to extract remaining lipids. After a final centrifugation at 10 000 × *g* for 2 minutes, supernatant was removed and pellets were air-dried. Proteins were analyzed by SDS-PAGE.

Protein homology modeling and molecular docking

Putative 3D-structures of murine BSPH1 and BSPH2 proteins and bovine BSP5 were predicted via an automated comparative protein modeling server (Swiss-Model) (http://www.expasy.ch) with the optimized mode using the coordinates of BSP1 (PDB accession number: 1H8P) available from the Brookhaven Protein Database (BPD) [38, 39]. Molecular docking analysis was performed using AutoDock-vina [40], and the ligand PDB coordinates were obtained from ChemSpider (http://www.chemspider.com). The electrostatic potential and molecular surface of each protein model was calculated through eF-surf server and visualized through PDBjViewer (version 3.0) [41].

Preparation of sperm

Male mice (between 10 and 24 weeks old) were sacrificed by cervical dislocation after which cauda epididymides and vas deferens were immediately removed. Tissues were cut four to six times in 1 ml pre-warmed modified Krebs-Ringer medium (Whitten's HEPES; WH; 100 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.5 mM D-glucose, 1 mM sodium pyruvate, 4.8 mM L(+)-lactic acid hemicalcium salt in 20 mM Hepes) buffered medium, pH 7.4 (osmolality 315 mOsm/kg) and placed at 37°C for 10 min to allow motile sperm to swim-out [42]. Motile sperm were collected, washed once with 5 ml of WH medium (10 min at 200 × *g*) and resuspended in 500 µl of WH medium.

Binding to sperm

7×10^6 sperm were incubated 1 h at 37°C in 1 ml of WH medium containing Complete Mini, EDTA-free protease inhibitor tablet (1 tablet/10 ml; Roche, Manheim, Germany) and 1 mM of PMSF. Sperm were either incubated alone, with 10 µg of rec-BSPH1 or 10 µg of rec-BSPH2. Following the incubation, sperm were washed with 1 ml of WH medium (10 min at 200× *g*). Cells were pelleted at 5000 × *g* for 10 min, the supernatant was removed and the pellet was washed twice with 1 ml of WH medium. The supernatant and wash fractions were precipitated with TCA (15%, final concentration), resuspended in Laemmli sample buffer and boiled 10 min. The pellet was resuspended in sample buffer, boiled 10 min and sonicated 1 hour in a Branson ultrasonic water bath (model 3510). The different fractions were analyzed by western blot.

Capacitation assay

Sperm were collected as described above. For capacitation studies, 2×10^6 washed sperm were incubated 1 h at 37°C in 1 ml WH medium in the presence of different concentration of rec-BSPH1 and/or rec-BSPH2, with 16 µg/ml of Trx-His-S, with 5 mg/ml of BSA or without any added proteins as control [35]. Following the incubation, 200 µl of the sperm suspension were incubated an additional 30 min at 37°C with or without 5 µM of calcium ionophore A23187 (Sigma-Aldrich). As controls, to test the effect of calcium ionophore on non-capacitated sperm, 2×10^6 sperm incubated in media without bicarbonate or BSA, therefore uncapacitated, were also treated with A23187 for 30 min. Sperm were then fixed with 200 µl of 8% paraformaldehyde for 30 minutes at room temperature, centrifuged for 2 min at 8000 × *g* and washed 2 times with 0.1 M ammonium acetate (pH 9.0). They were finally resuspended in a final volume of 100 µl of the same solution and 20 µl were smeared on microscopic slides. Slides were dried and stained using the Coomassie Brilliant Blue staining technique [43]. Briefly, slides were placed successively in water, methanol and water for 5 minutes each and stained 2 minutes in a solution of 0.22 % Coomassie Blue G-250 in 50% methanol and 10% acetic acid solution. 400 sperm were counted for each condition.

Immunolocalization of rec-BSPH2 on sperm

Cauda epididymides were placed in 1 ml of Human Tubal Fluid (HTF) medim (101.6 mM NaCl, 4.7 mM KCl, 0.37 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 2.78 mM glucose, 0.33 mM pyruvate, 21.4 mM lactate, 100 U/ml of penicillin G, and 0.1 mg/ml of streptomycin, osmolality of approximately 315 mOsm/kg) [44] and cut 4 to 6 times with scissors. Sperm were dispersed in the medium for 10 minutes at 37°C under 5% CO_2. Tissues were removed and sperm were dispersed by gentle swirling.

Two million sperm were incubated 1 h in HTF medium without any added proteins, with 15 µg rec-BSPH2, 15 µg rec-BSPH1 or 7 µg Trx-His-S as control in the presence or absence of BSA. Following incubation with the recombinant proteins, 200 µl of the sperm suspension were incubated an additional 30 min at 37°C with or without 5 µM of calcium ionophore A23187 and fixed with 200 µl of 4 % paraformaldehyde for 30 min at room temperature. The sperm were then washed 3 times with PBS and allowed to dry on Poly-L-lysine microscopic slides (Fisher Scientific, Ottawa, ON, Canada). Sperm were permeabilized for 5 minutes with PBS containing

0.1 % Triton-X-100 and 0.2 % paraformaldehyde, washed 3 times with PBS and blocked for 1 h at room temperature in PBS-1 % BSA. Slides were then incubated 1 h at room temperature with a mix of anti-15mer and anti-BSPH1 antibodies (dilution, 1:400 each) or mouse His-probe antibodies (dilution, 1:100) diluted in PBS-0.1 % BSA. They were washed three times with PBS-0.1% BSA to remove excess antibodies and incubated 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) at a dilution of 1:200 in PBS-0.1 % BSA. After three final washes with PBS, slides were mounted with DABCO 1.5% (10 ml 15% DABCO and 90 ml glycerol).

For the double staining, tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Sigma-Aldrich) at a dilution of 1:200 was used as secondary antibodies. Slides were washed with PBS and each slide was incubated for 30 min in the dark with a droplet of 75 µg/ml of *Pisum sativum* agglutinin conjugated to fluorescein isothiocyanate (PSA-FITC, Sigma) diluted in PBS. Slides were washed three more times with PBS and mounted with DABCO 1.5%. Observations were done under a fluorescence microscope (Zeiss Axio Imager).

Statistical Analysis

Data are presented as the mean ± S.E.M. Differences 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

Cloning, expression and purification

Rec-BSPH2 was expressed in Rosetta-gami B(DE3)pLysS, denatured with urea and purified by IMAC. Proteins were refolded on-column slowly using a decreasing urea gradient [35]. Most of the bound proteins were eluted using 250 mM imidazole (E2) (Fig. 1A). Following that elution, very little proteins remained in the column. Analysis of the different purification fractions by SDS-PAGE (Fig. 1B) revealed the presence of a band of ~32 kDa in every fraction. 32 kDa is the predicted molecular weight of rec-BSPH2 (20 kDa for Trx-His-S and 12 kDa for BSPH2). In western blot analysis, the band predicted to be rec-BSPH2 was recognized strongly by His-probe monoclonal antibodies and cross-reacted with anti-BSPH1 antibodies (data not shown). LC-MS/MS analysis of the band confirmed the identity of BSPH2 protein (Fig. 1D).

SDS-PAGE of fraction E2 under non-reducing conditions (Fig. 1C) revealed the presence of many bands of higher molecular weight. These bands, recognized by His-probe monoclonal antibodies were identified as oligomers of rec-BSPH2. Fraction E1 (elution with 70 mM imidazole) contained a high amount of impurities and fraction E3 (400 mM imidazole) contained only traces of proteins. Therefore, in the current study, protein in fraction E2 desalted on a Sephadex G-25 column in 0.05 M ammonium bicarbonate and lyophilized was used. From 1 liter of culture, ~15-20 mg of pure protein was obtained in each run.

Binding properties

Interaction of rec-BSPH2 with gelatin, heparin, CSB was tested by affinity chromatography. Rec-BSPH2 was found to interact with gelatin and heparin but not CSB. On a gelatin-agarose affinity chromatography column (Fig. 2A), 45-50% of the total protein was retained and eluted with 8 M urea. Interaction was stronger with heparin as more than 95% of the proteins required 1 M NaCl to be eluted from the heparin-sepharose column (Fig. 2B). In contrast, on CSB-agarose affinity column (Fig. 2C), rec-BSPH2 was found in majority (~ 92%) in the unabsorbed fraction. These results are representative of at least three separate experiments. As control, the binding of bovine BSP proteins was also tested (data not shown). Bovine BSP proteins bound strongly to all three resins.

Interaction with liposomes made of PC, PC/PE/PI or PC/CHO was tested using ultracentrifugation methods (Fig. 3A). In all conditions tested, the recombinant protein was found in the supernatant fraction. A more acidic pH during incubation did not alter the results (not shown). On the other hand, the bovine BSP1, which binds strongly to the phospholipids, was found in the pellet fraction when incubated with liposomes.

Finally, the binding of rec-BSPH2 to epididymal sperm was tested (Fig. 3B). Epididymal sperm were incubated for one hour alone, with 10 µg of rec-BSPH1 or 10 µg of rec-BSPH2, and sperm extracts were analyzed by western blot using anti-BSPH1 antibodies. Analysis of the epididymal sperm incubated in the absence of any recombinant protein revealed the presence of a band at ~14 kDa, the expected molecular weight of both native BSPH1 and native BSPH2. As previously observed [35], when rec-BSPH1 was incubated with the epididymal sperm, most of the proteins were found in the pellet with sperm. A signal was also observed in the supernatant, indicating that a fraction of the recombinant protein did not bind to the sperm. A third band at

~16 kDa was observed in the pellet as well. This band was sequenced and identified as rec-BSPH1 with parts of its Trx-His-S tag missing. Finally, for rec-BSPH2 incubated with epididymal sperm, two bands were observed; a faint band at 32 kDa in the supernatant fraction and a band at 14-16 kDa in the sperm pellet. This band was identified as BSPH2 and could be either the native BSP proteins or rec-BSPH2 without the Trx-His-S tag or a mixture of both proteins. However, since the signal of this band is much stronger than the one usually observed for the native BSP proteins, a mixture of both proteins is most probable. No clear 32 kDa band was observed in the pellet.

In silico characterization, homology modeling and molecular docking

Since Rec-BSPH1, rec-BSPH2 and native bovine BSP proteins are found in different oligomeric forms, predicted models of the proteins homodimeric structures were analyzed. The electrostatic potential for each predicted dimeric forms of BSP1, BSP5, BSPH1 and BSPH2 was calculated. Results show that the surface of predicted gelatin-binding sites is mostly hydrophobic (yellow) or ionic positive (blue) (Fig. 4D-G). Results also show that, in the dimer, gelatin-binding sites are arranged on the same side (Fig. 4C).

Molecular docking analysis of BSPH1 and BSPH2 proteins with PC was also performed to support the *in-silico* evidence that rec-BSPH2 does not bind to PC liposomes (Fig. 5). In the first Fn2 domain (D1), the binding affinity between both proteins is similar (-3.8 kcal/mol), but all the top 9 conformations in BSPH1 are within the ligand-binding pocket, whereas there are only four conformations in BSPH2. For the second Fn2 domain (D2), BSPH1 is predicted to bind PC with an affinity of -3.5 kcal/mol. Top conformations in BSPH2 are not located within its binding pocket and the one that does has an affinity of -2.8 kcal/mol. This shows that BSPH1 has stronger binding affinity for PC than that of BSPH2 in general.

Immunolocalization

Localization of rec-BSPH2 on epididymal sperm was evaluated and compared to the immunofluorescence pattern of rec-BSPH1. Sperm incubated with rec-BSPH2 in the presence or absence of BSA, had a faint signal over the midpiece region and a stronger signal over the head (Fig. 6A). This binding pattern is very similar to the one observed for rec-BSPH1. However, rec-BSPH1 was found over the midpiece of the sperm only when BSA was present in the incubation

medium. In the absence of recombinant proteins (Fig. 6B), although the signal was very faint, the native proteins were observed over the head of the sperm. Trx-His-S did not bind to the sperm as previously shown [35]. No signal was observed for this control.

At higher magnification, depending on the cell examined, two different fluorescence patterns were observed over the head of the sperm. However, the patterns were similar for both rec-BSPH1 and rec-BSPH2. This was investigated further using double staining. PSA-FITC was used to assess the state of the sperm (uncapacitated, capacitated or acrosome reacted) and a mixture of anti-15mer/anti-BSPH1 antibodies was used to mark the BSP proteins. PSA-FITC staining over the anterior acrosome region indicated that sperm were uncapacitated (Fig. 7A-B), whereas faint PSA-FITC staining over the anterior acrosome and strong staining over the equatorial segment indicated capacitated sperm (Fig. 7C-D). On uncapacitated sperm both rec-BSPH1 (Fig. 7A) and rec-BSPH2 (Fig. 7B) had similar binding patterns and were found over the anterior- and post-acrosomal regions. Following capacitation, rec-BSPH2 (Fig. 7D) was found only over the equatorial segment whereas rec-BSPH1 (Fig. 7C) was found over both the anterior acrosome region and the equatorial segment. On acrosome reacted sperm (PSA-FITC only over the equatorial segment), the fluorescence pattern was the same as when sperm were capacitated (not shown).

Effect on sperm capacitation

Generally, only capacitated sperm can undergo the acrosome reaction (AR). In this set of experiments, capacitation was assessed by the ability of sperm to undergo acrosome reaction (AR) induced by calcium ionophore A23187 (Fig. 8A). Without recombinant proteins or BSA, the basal level of AR in the presence or absence of A23187 was 28 ± 3 %. Level of AR for uncapacitated sperm in the presence of A23187 was similar at 25 ± 1 %. In the presence of 5 mg/ml of BSA (capacitation condition), the addition of A23187, stimulated the level of AR up to 72 ± 7 %. None of the rec-BSPH2 concentrations tested increased the level of AR. As previously shown, the addition of increasing concentrations of rec-BSPH1 caused a dose-dependent increase in the levels of acrosome reacted sperm [35]. The addition of rec-BSPH1 and rec-BSPH2 at the same time had no impact on the level of AR (Fig. 8B). Similarly, co-incubation of sperm with BSA and rec-BSPH2 had no impact on the level of AR (not shown). Levels of AR obtained when both proteins were present were comparable to the levels obtained for rec-BSPH1 alone at similar

concentrations. For all conditions tested, without the addition of A23187, the levels of acrosome reacted sperm remained at the basal level (Not shown).

DISCUSSION

Expression and purification of rec-BSPH2

Native BSP proteins are found in minute quantities in mice. For this reason, it was necessary to produce recombinant proteins to study their functions. One of the challenges encountered during the production of recombinant BSP proteins is the formation of the four disulfide bridges. To overcome this problem, a bacterial system combining the pET32a expression vector with *E. coli* Rosetta-gami B(DE3)pLysS cells was used. This system has been proven to be ideal to produce recombinant proteins containing disulfide bridges [35, 45-48]. Since BSPH2 is not predicted to be glycosylated or to contain any major modifications [17], the lack of machinery necessary for post-translational modification in prokaryotes should not affect the function of rec-BSPH2. However, this system alone is not sufficient with BSP proteins to produce non-aggregated, soluble proteins. Following expression, proteins were purified by IMAC and refolded on column using a decreasing urea gradient [35]. With this method, a good yield of pure, soluble rec-BSPH2 protein was obtained. Under non-reducing condition, rec-BSPH2 was found in several levels of oligomerization a phenomenon observed with native BSP proteins in bulls, boars and stallions [20, 28, 49]. Both murine BSP proteins share 34% identity and 55% similarity of sequence and have very similar C-terminal tails [17]. Results show that they also share antigenic sites as rec-BSPH1 and rec-BSPH2 cross-reacted with the same antibodies (anti-BSPH1 and anti-15mer), but with different affinity. Anti-BSPH1 antibodies were used for the subsequent analysis.

Binding to GAGs and gelatin

Bovine BSP proteins and their homologs, in view of their structural similarities, share many binding characteristics such as binding to gelatin and GAGs [20, 24, 28, 30-32, 49-51]. In the present study, results show that rec-BSPH2 binds to GAGs and gelatin similarly to rec-BSPH1 or human recombinant BSPH1 [35]. It could bind to heparin but not CSB. Proteins can interact with more than one GAG but with different affinities [52]. Binding to heparin has been attributed to the interaction of basic amino acids, mostly lysine and arginine, with negative charges of heparin [53]. Studies have demonstrated that the preferred motifs in heparin binding proteins are

XBXBX, XBXXBX and XBXXXBX where "B" represents basic amino acid(s) and "X"; any amino acid [53]. BSPH2 sequence contains 9 of these motifs. Binding to CSB has not been studied as thoroughly as heparin. For many proteins, the exact binding site has not been identified [52]. The exact reason for the lack of interaction between rec-BSPH1 or rec-BSPH2 and CSB is unknown.

Binding to gelatin is attributed to the presence of the two Fn2 domains. In addition to an appropriate 3D structure, it has been shown that some amino acids are essential for gelatin binding. In the Fn2 domain of human gelatinase A/MMP-2, replacement of the tyrosine by an arginine in position 37 can completely abolish the gelatin-binding properties of the protein without destroying its 3D conformation [54]. Therefore, amino acid replacements were examined on aligned sequences of BSPH1, BSPH2 and bovine BSP5 as well as on predicted models of the BSP proteins (Fig. 4A-C). The first Fn2 domain of BSPH2 was found to be the only one with a tyrosine in that position. Since BSP5 can bind strongly to gelatin, and has no tyrosine in the corresponding position (Y37), this particular amino acid probably is not essential for the gelatin binding of BSP proteins.

Gelatin is a derivative of collagen, a principal structural and connective protein in animals. Hydrophobicity can be an indicator of regions on a protein surface that are involved in protein-protein interaction [55]. Homology modeling revealed that the predicted gelatin-binding sites of dimeric forms were arranged on the same side of the dimer suggesting that BSP proteins could bind gelatin as a dimer through this side of the molecules (Fig 4C). Furthermore, it suggests that oligomerization could be necessary for a strong binding as it has been shown for heparin binding in equine, porcine and bovine BSP proteins [27, 28, 56]. Differences in oligomerization could explain the reduced binding of the recombinant proteins to the gelatin-agarose beads. It is also possible that part of the recombinant proteins were not folded in the appropriate 3D structure. Nevertheless, the ability of the BSP proteins to bind to gelatin is not linked with its role in capacitation. It should be noted that rec-BSPH1 has similar binding properties and could promote murine sperm capacitation [35].

Binding to phospholipid liposomes

Proteins of the BSP superfamily are known to interact with sperm membrane mainly via choline phospholipids [21, 28-30]. Surprisingly, rec-BSPH2 does not bind to PC liposomes

despite the fact that it can bind to murine epididymal sperm. The binding of BSP proteins with PC is due to a cation interaction between the amine group of the choline and a tryptophan residue of the Fn2 domains, as well as hydrogen bonds between six tyrosine residues of the protein and phosphate groups of the PC [57]. Three of those six tyrosine residues are missing in the BSPH2 sequence (Fig. 4A, blue stars above), which could explain the low affinity of the protein for PC. Since no sperm proteins are known to interact with BSP proteins, interaction with other lipidic component of the plasma membrane was investigated.

The BSPH2 shows more sequence similarities with the bovine BSP5 protein, the only bovine BSP that can bind to PE, PI and phosphatidylserine as well as PC [16, 17, 21]. Furthermore, some studies have shown an increase in the association constant of bovine BSP1 with PC liposomes when they contained cholesterol [58]. It was suggested that this interaction could be due to the presence of CRAC (Cholesterol Recognition Amino acid Consensus) domains in the BSP structure [59]. Epididymal sperm membranes are made mainly of choline containing phospholipids (67-70%) but also contain PE (19-22%), PI (9-10%), and cholesterol (CHO: lipid ratio ~0.24 and 0.29) [60]. The addition of PE, PI or cholesterol to PC liposomes in similar proportions did not influence the affinity of rec-BSPH2 for the liposomes.

Molecular docking analysis confirmed that BSPH1 has a strong affinity for PC, whereas BSPH2 has low binding affinity. Thus, rec-BSPH2 could be interacting with the choline containing phospholipids on sperm membrane much more weakly than other BSP proteins. It is possible that the interaction was too weak to be observed using PC liposomes or that the organization of the lipids in the liposomes was not optimal for the rec-BSPH2 binding.

Immunofluorescence

It was previously demonstrated that, when incubated with epididymal sperm, rec-BSPH1 is found mostly over the head of the sperm, more specifically over the anterior acrosome and the equatorial segment, and that, in the presence of BSA, proteins can be found over the midpiece as well [35]. In this study, rec-BSPH2 was found over the head of the sperm and the midpiece in the presence or absence of BSA. Results also suggested a re-localization of both rec-BSPH1 and rec-BSPH2 during sperm maturation, which could be due to redistribution of phospholipids on the external surface of the sperm membrane [61].

On uncapacitated sperm, both recombinant BSP proteins were found on the anterior acrosome region as well as the post-acrosomal region. Proteins binding to the acrosome region of the sperm are often implicated in sperm capacitation. The post-acrosomal region has been shown to contain low levels of cholesterol when compared to the anterior acrosome and the equatorial segment [61]. Since cholesterol is known to add rigidity to plasma membrane and stabilize its structure, binding of BSPH1 and BSPH2 could compensate for the low levels of cholesterol in this region. This is in accordance with the hypothesis that, in mice, BSP proteins bind to the sperm in the epididymis to prevent free movements of the phospholipids in the membrane, thus preventing premature capacitation [35]. Following capacitation and acrosome reaction, both proteins were found over the equatorial segment. This segment of the sperm is implicated in the fusion of the sperm with the egg membrane, which suggests a possible role in sperm functions other than capacitation [62]. Since both recombinant proteins bind over the midpiece region, an additional role for murine BSP proteins in sperm motility is possible. Indeed, the midpiece region of sperm contains mitochondria and is often associated with sperm motility. Murine BSP proteins may play a role in motility like bovine BSP1, which has been shown to stimulate sperm motility and the activity of membrane-bound calcium ATPase in bull sperm [26, 63].

Effect on sperm capacitation

The main function associated with BSP proteins is their ability to promote sperm capacitation. BSP in bovine, boar and human, as well as rec-BSPH1 have been shown to induce sperm capacitation [23, 34, 35]. The fact that rec-BSPH2 cannot promote sperm capacitation sets it apart from the other members of the BSP superfamily. Current models suggest that BSP proteins are able to promote sperm capacitation by mediating a phospholipid/cholesterol efflux [24, 35]. Since BSPH2 interacts with PC with a much lower affinity than BSPH1, it is possible that the interaction is too weak for BSPH2 to extract phospholipids and cholesterol from the membrane to promote capacitation.

In conclusion, results in this study reveal major biochemical and functional differences between rec-BSPH2 and other proteins of the BSP superfamily. Rec-BSPH2 was able to bind to epididymal sperm similar to other BSP superfamily proteins. However, it is the first member of the BSP family that neither binds to PC liposomes nor promotes sperm capacitation. Furthermore, the results provide the first evidence that two BSP proteins from the same species do not seem to

have redundant functions. In view of this, BSPH2 is distinct and indeed is the black sheep of the BSP superfamily. Further work is warranted to establish whether BSPH2 might be involved in other steps of the fertilization process such as sperm egg-interaction or sperm-egg fusion. These results are a key step in delineating the redundancy of the BSP proteins with respect to their different characteristics and biological roles.

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FIGURE LEGENDS

Fig. 1. *Expression, purification and on-column refolding of rec-BSPH2.* (A) IMAC profile for the stepwise elution of rec-BSPH2 protein. (B) Analysis of the column fractions (reducing condition) by 15% SDS-PAGE stained with Coomassie Blue. P, pellet of the cell lysate; I, input; FT, flow through; NA, non-adsorbed; W, wash; E1 (tubes 9-46), elution with 70 mM imidazole; E2 (tubes 47-65), elution with 250 mM imidazole; E3 (tubes 79-92), elution with 400 mM imidazole. Approximately, 15 μg proteins were loaded in each lane except lane E2 where 4 μg proteins were loaded. The pellet (P) of the cell lysate, was resuspended in sample buffer and 1/1000 of the fraction was loaded in lane P. (C) Analysis of fraction E2 on 12% SDS-PAGE in non-reducing condition. (D) LC-MS/MS analysis of the 32 kDa band in fraction E2. Bold letters represent residues identified by LC-MS/MS.

Fig. 2. *Binding of rec-BSPH1 to gelatin and GAGs.* Affinity chromatography profiles of rec-BSPH2 on (A) gelatin-agarose, (B) heparin-sepharose and (C) chondroitin sulfate B-agarose columns. Approximately 500 μg (gelatin and CSB) or 5 mg (heparin) of proteins were loaded on each column. The columns were then washed with 50 mM TB, and eluted with TB containing 1 M NaCl (GAGs) or 8 M urea (gelatin columns).

Fig. 3. *Phospholipid liposomes- and mouse caudal sperm-binding assay*. (A) 20 μg of rec-BSPH1 and 10 μg of bovine BSP1 were incubated with or without 300 μg of PC, PC/PE/PI or PC/CHO liposomes and then centrifuged. The supernatant (S) and pellet (P) were separated and analyzed by western blot using mouse anti-BSPH1 and bovine anti-BSP1 antibodies respectively. (B) Caudal sperm were incubated alone, with 10 μg of rec-BSPH1 or 10 μg of rec-BSPH2 and then centrifuged. The supernatant (S) was removed and the sperm pellet was washed. The wash fraction (W) and the sperm pellet (P) were separated by centrifugation and equivalent proportions of each fraction were analyzed by western blot using mouse anti-BSPH1 antibodies.

Fig 4. *Sequence comparison and homology modeling of BSPH1 and BSPH2 and predicted gelatin-binding sites along a dimeric structure.* (A) Predicted amino acid sequence alignment of BSPH1, BSPH2 and BSP5. The triangle indicates the conserved cycteine

residues. Arrow below the alignment represents the position of the amino acid that was previously demonstrated to be essential for gelatin binding [54]. Sites predicted to bind PC are indicated on the top of the alignment by asterisks (*)[57]. (B) 3-D homology modeling of BSPH1, BSPH2, and BSP5 based on the BSP1 template. (C) Dimeric structure of BSPH1 shows that the critical amino acids (site) responsible for gelatin-binding (in purple) are along the same side with another monomer. (D-G) Electrostatic potential surface colored from red (negative) to blue (positive) and yellow (hydrophobic) are shown on each active site surface of BSPH1, BSPH2, BSP5 and BSP1 in a dimeric structure.

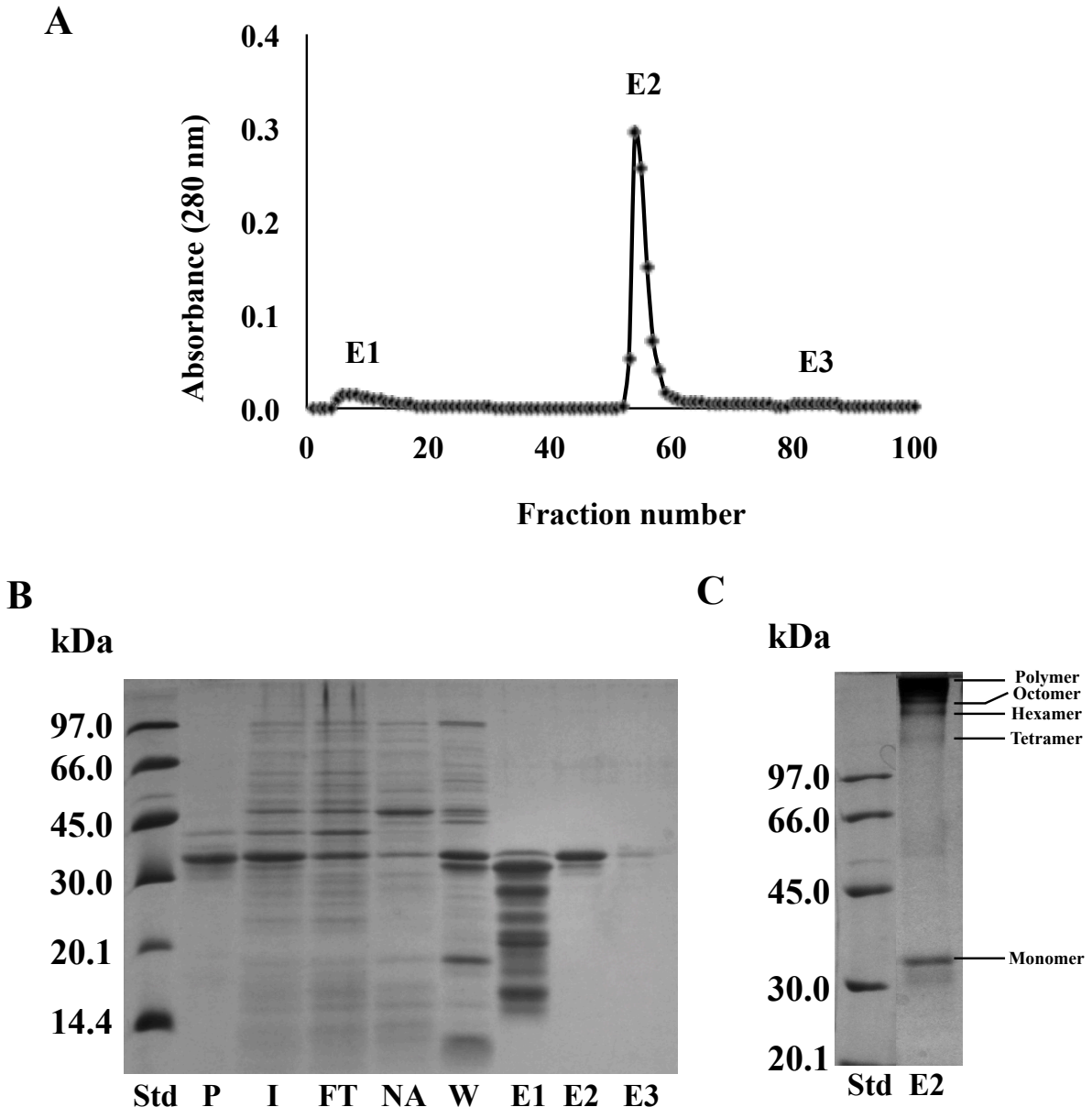
Fig. 5. Molecular docking analysis on the homology models of BSPH1 and BSPH2 with their potential ligand: phosphorylcholine ($C_5H_{15}C_lNO_4P$; PC). Ribbon cartoon representative structure of BSPH1 and BSPH2 in docking with PC in Van der Waals spheres on its Fn2-domain 1 (D1) (A and C respectively) and Fn2-domain 2 (D2) (B and D respectively). A list of top 9 conformations of the ligand docking with its receptor(s) in ranking of binding affinity are shown below each panel as kcal/mol. The top conformation of the ligand within the potential binding pocket is highlighted in rectangular dotted line. Other similar conformations are indicated by red arrows. The root-mean-square deviation lower /upper bound (RMSD l.b. / u.b.) values are provided.

Fig. 6. *Immunostaining of rec-BSPH2 on cauda epididymal sperm.* (A) Sperm collected from cauda epididymis were incubated in medium containing 15 μg rec-BSPH1 or 15 μg rec-BSPH2 with (bottom panel) or without (top panel) 5 mg/ml BSA. Slides were incubated with a mix of anti-15mer and anti-BSPH1 antibodies at dilution of 1:400 each and treated with FITC-conjugated IgG. (B) For detection of native proteins, cauda epididymal sperm were incubated without recombinant proteins, smeared on slides and incubated with the mix of anti-BSPH1/anti-15mer. As control, cauda epididymal sperm were incubated with 7 μg Trx-His-S and smeared on slide and incubated with His-probe antibodies at dilution of 1:200. All slides were then treated with anti-mouse FITC-conjugated IgG (Original magnification × 630).

Fig. 7. *Binding patterns of rec-BSPH1 and rec-BSPH2 on caudal sperm.* Sperm collected from mouse epididymides were incubated with 15 µg of rec-BSPH1 or 15 µg of rec-BSPH2 in HTF medium alone, supplemented with BSA or containing both BSA and calcium ionophore A23187. Sperm were then fixed with 4% (w/v) paraformaldehyde and smeared on slides. Slides were incubated with a mix of anti-BSPH1/anti-15mer antibodies and treated with TRITC-conjugated goat-anti-rabbit IgG. Slides were then incubated with PSA-FITC (Original magnification × 1250). Double staining of the sperm showed different patterns of binding when sperm were uncapacitated (A, B) or capacitated (C, D) for rec-BSPH1 and rec-BSPH2 respectively.

Fig. 8. *Effect of rec-BSPH2 on murine sperm capacitation.* Capacitation was assessed by the ability of sperm to undergo the AR induced by A23187 ionophore. Epididymal sperm were incubated with different concentrations of rec-BSPH1, rec-BSPH2, 32 µg/ml of Trx-His-S or 5 mg/ml of BSA (A) or different concentrations of rec-BSPH1 and rec-BPSH2 (B) for 60 min followed by incubation with calcium ionophore A23187 for 30 min. Sperm were smeared on slides and analyzed by Coomassie Blue staining. A minimum of 400 sperm per conditions were evaluated. Data are means ± S.E.M for four independent experiments. Differences compared to control (sperm alone) were analyzed by one-way ANOVA followed by Bonferroni post hoc test. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

FIGURE 1



D

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  1 ELISHLHPPE QEISTDSCVF PFVYADGFHY SCISLHSDYD WCSLDFQFQG
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101 WKQCSPNNF
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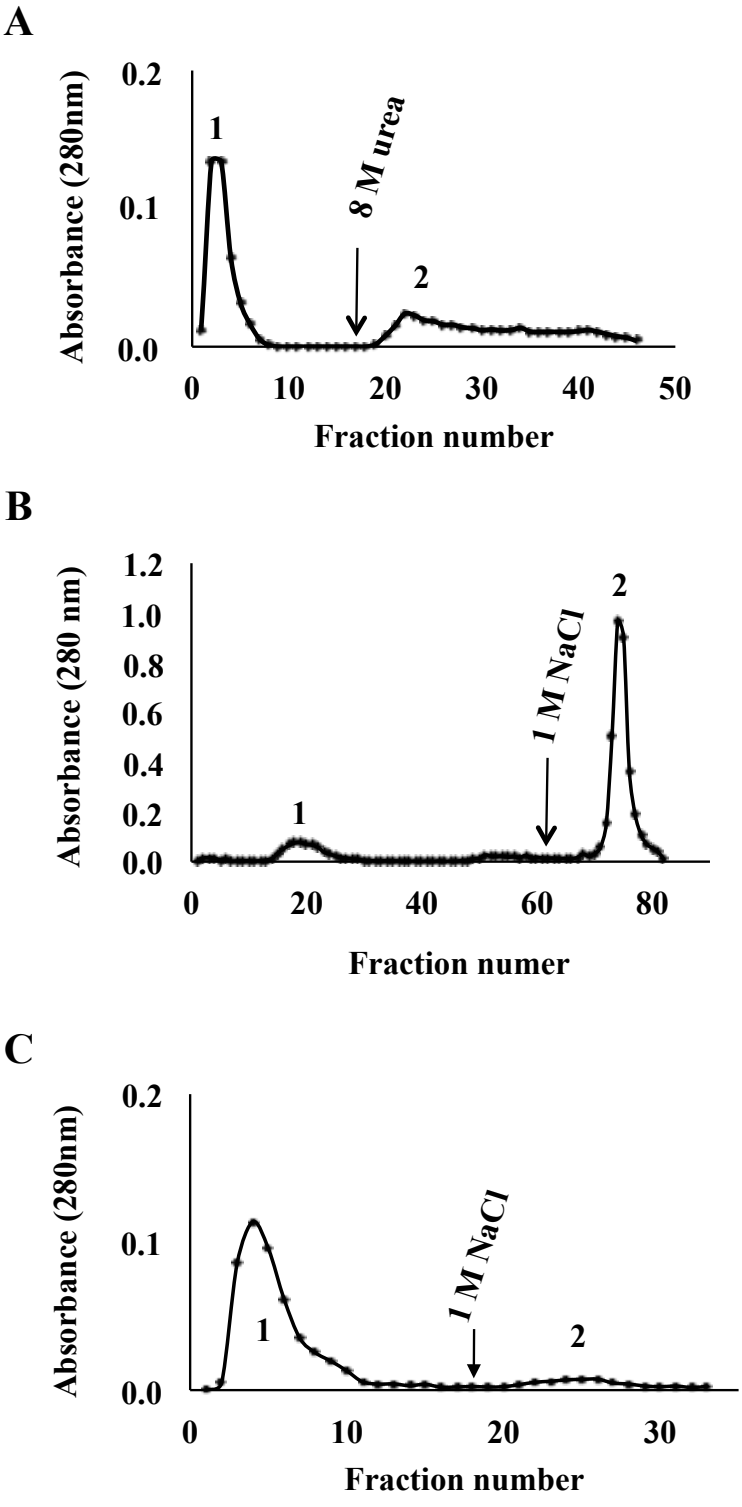
FIGURE 2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Absorbance (280nm)
0.2
0.1
0.0
1
8 M urea
2
0
10
20
30
40
50
Fraction number
B
Absorbance (280 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
1 M NaCl
2
0
20
40
60
80
Fraction numer
C
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1
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2
0
10
20
30
Fraction number

FIGURE 3

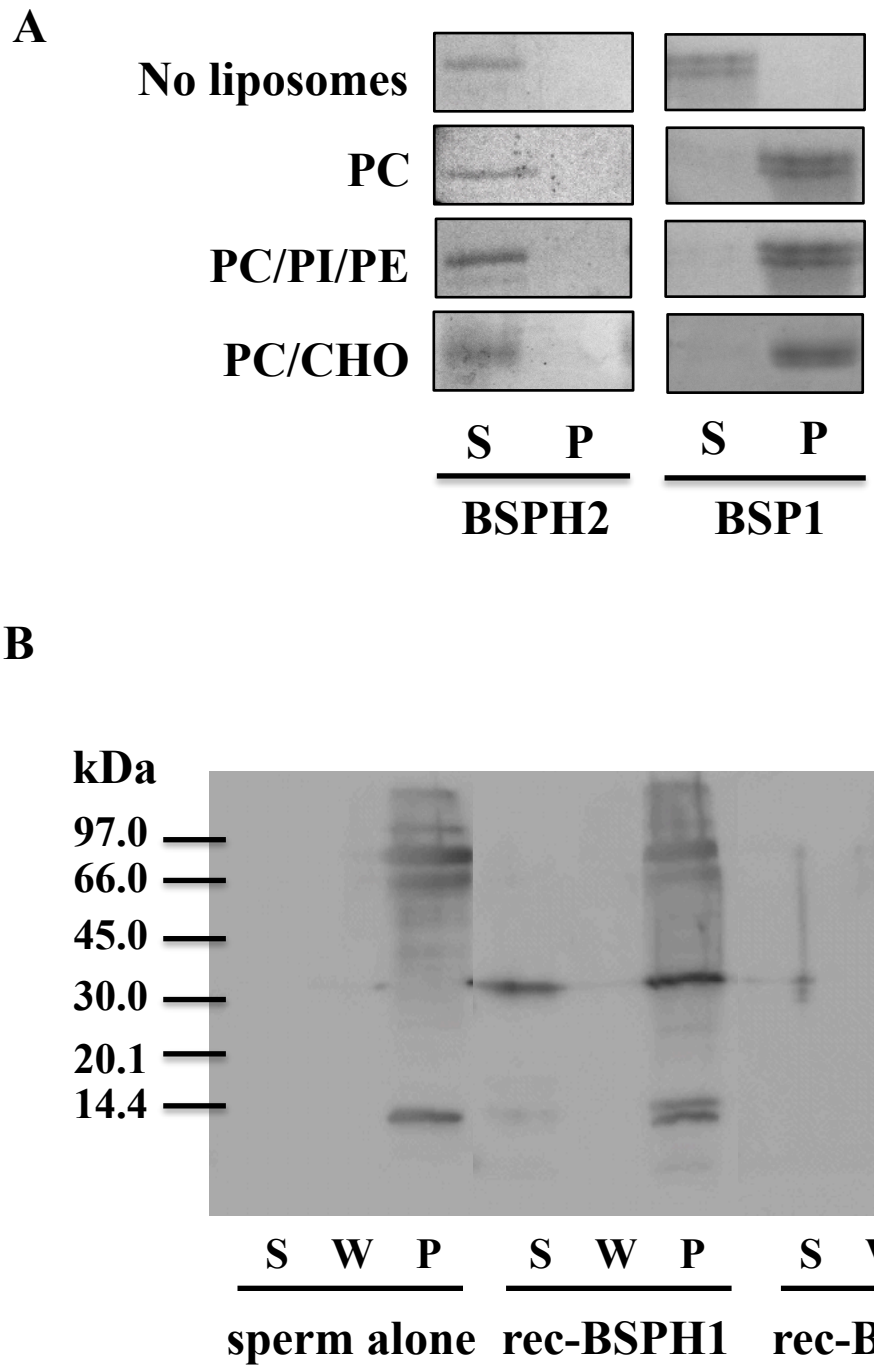

A
No liposomes
PC
PC/PI/PE
PC/CHO
S P S P
BSPH2 BSP1
B
kDa
97.0
66.0
45.0
30.0
20.1
14.4
S W P S W P S W P
sperm alone rec-BSPH1 rec-BSPH2

FIGURE 4

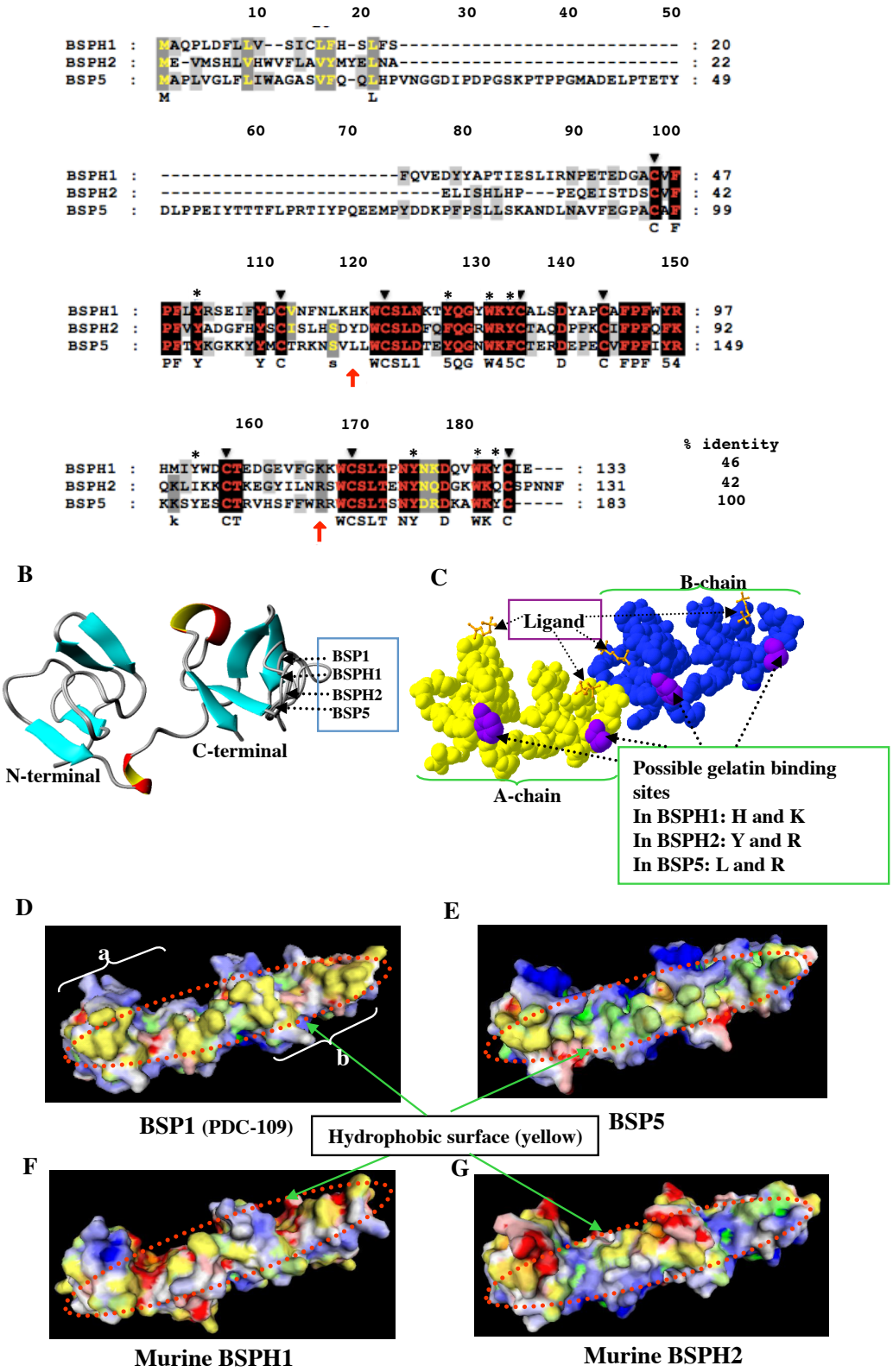
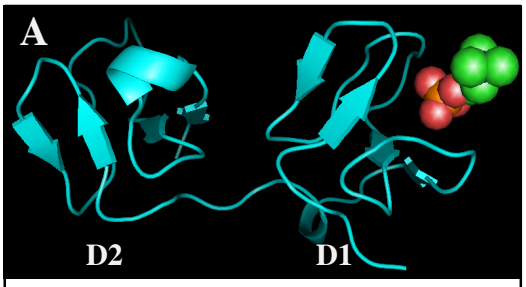

A
B
C
D
E
F
G
BSP1
BSPH1
BSPH2
BSP5
% identity
46
42
100
N-terminal
C-terminal
B-chain
Ligand
A-chain
Possible gelatin binding sites
In BSPH1: H and K
In BSPH2: Y and R
In BSP5: L and R
a
b
BSP1 (PDC-109)
Hydrophobic surface (yellow)
BSP5
Murine BSPH1
Murine BSPH2

FIGURE 5



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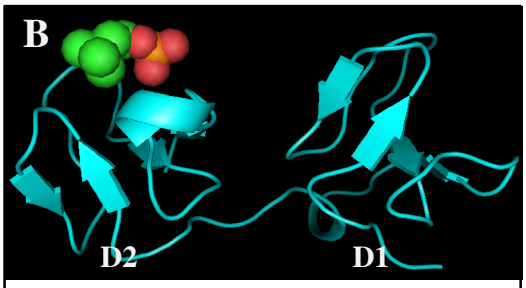
3	-3.6	3.639	4.604
4	-3.6	2.181	3.024
5	-3.5	3.784	4.825
6	-3.5	2.585	3.656
7	-3.4	2.320	3.425
8	-3.3	1.193	2.314
9	-3.1	2.731	3.445



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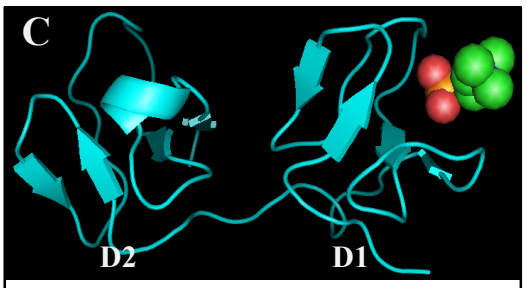
3	-3.2	15.372	16.125
4	-3.1	15.223	16.090
5	-3.1	14.015	14.887
6	-3.0	15.258	16.409
7	-3.0	16.835	17.781
8	-3.0	14.004	14.949
9	-3.0	14.192	15.169



mode :	affinity : (kcal/mol) :	dist from best mode rmsd l.b. :	rmsd u.b.
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3	-3.6	1.822	2.584
4	-3.1	9.160	10.139
5	-3.0	8.350	9.337
6	-3.0	16.379	17.144
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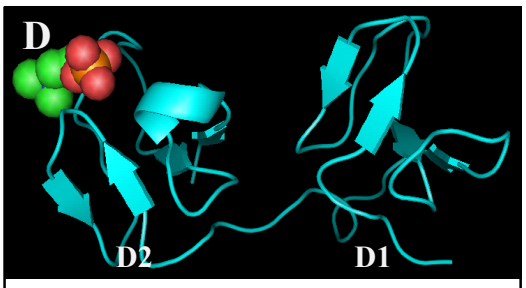
9	-3.0	2.733	3.419



mode :	affinity : (kcal/mol) :	dist from best mode rmsd l.b. :	rmsd u.b.
1	-3.1	0.000	0.000
2	-3.0	11.887	12.915
3	-2.9	10.491	11.807
4	-2.9	21.345	22.067
5	-2.9	1.224	2.301
6	-2.9	9.914	11.101
7	-2.8	19.098	20.035
8	-2.7	3.264	4.106
9	-2.7	21.557	22.324

FIGURE 6

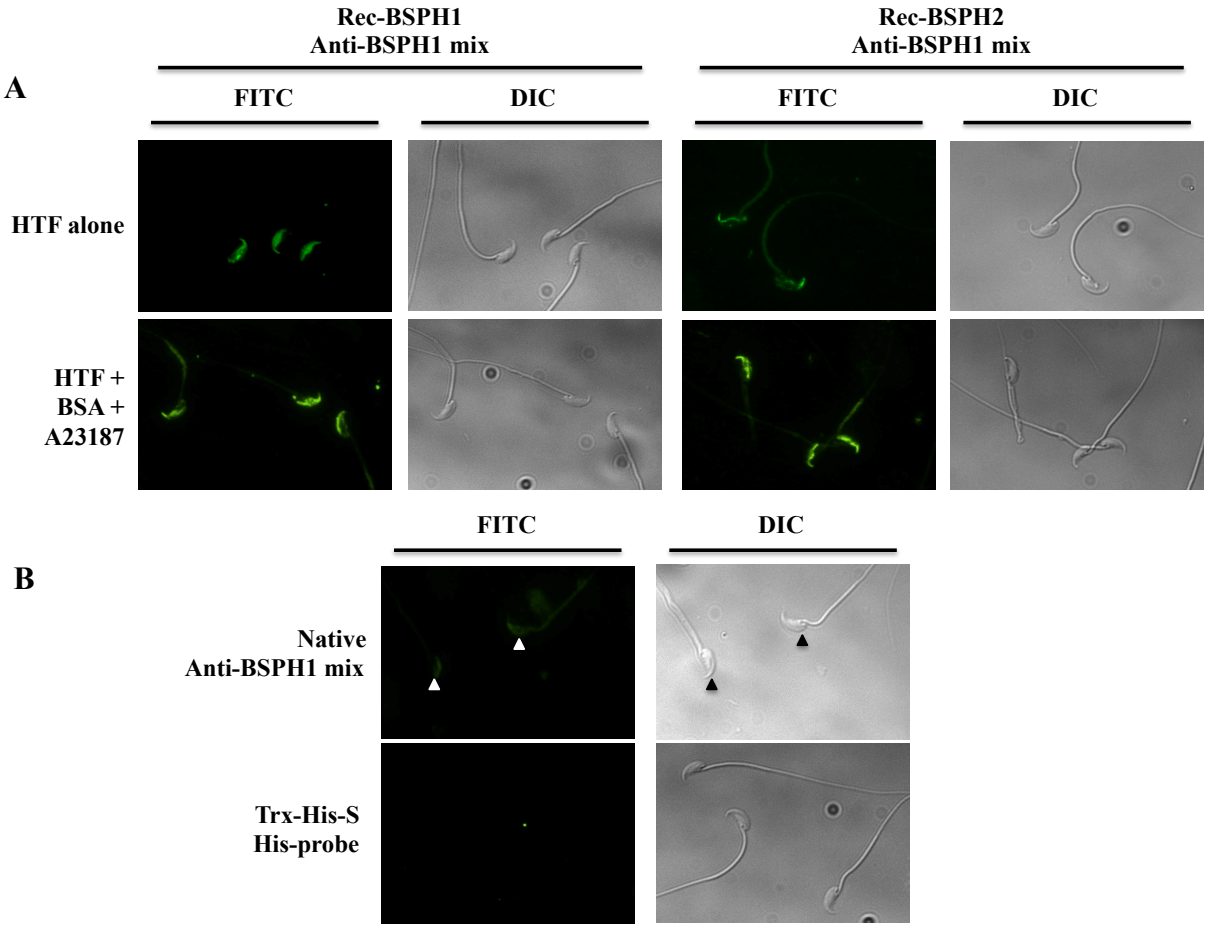

A
Rec-BSPH1
Anti-BSPH1 mix
Rec-BSPH2
Anti-BSPH1 mix
FITC
DIC
FITC
DIC
HTF alone
HTF +
BSA +
A23187
B
FITC
DIC
Native
Anti-BSPH1 mix
Trx-His-S
His-probe

FIGURE 7

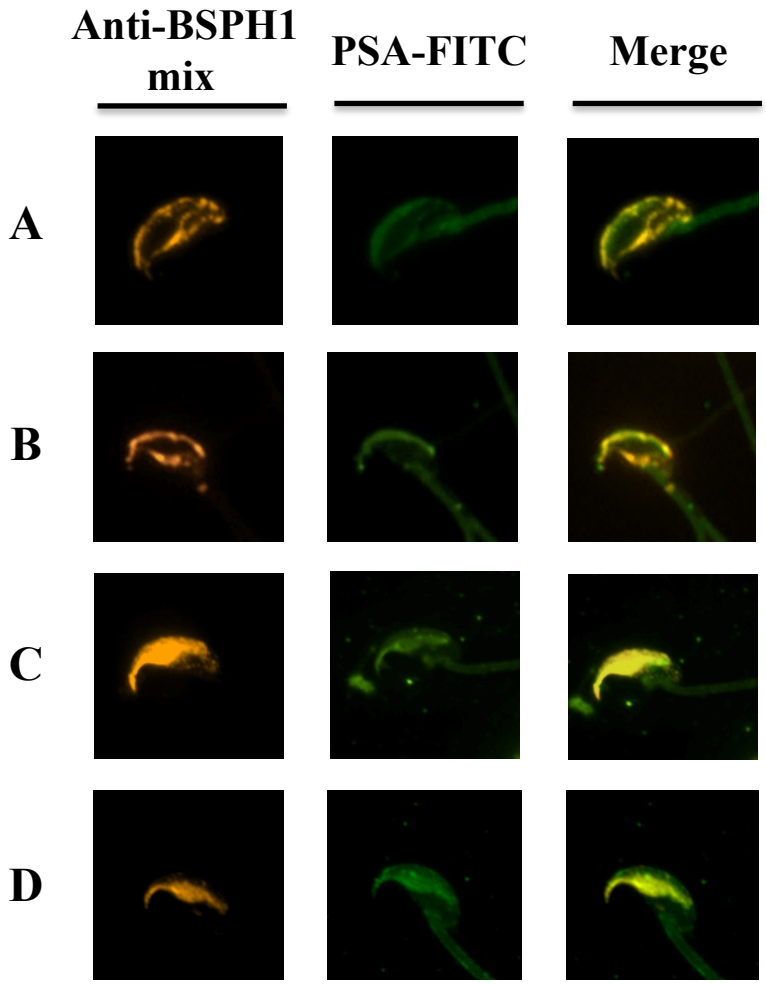
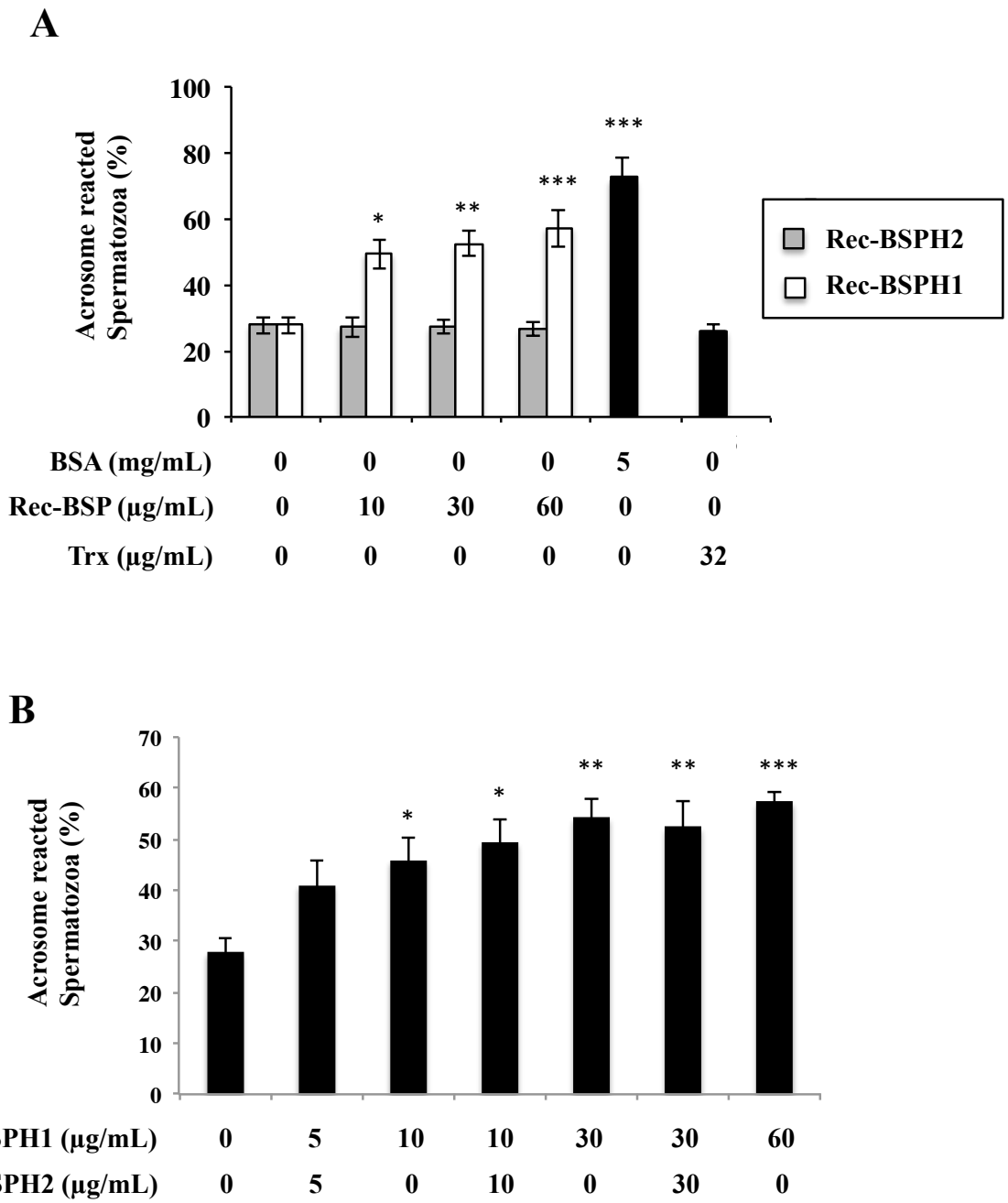


FIGURE 8


A
Acrosome reacted Spermatozoa (%)
100
80
60
40
20
0
*
**
***
***
Rec-BSPH2
Rec-BSPH1
BSA (mg/mL) 0 0 0 0 5 0
Rec-BSP (µg/mL) 0 10 30 60 0 0
Trx (µg/mL) 0 0 0 0 0 32
B
Acrosome reacted Spermatozoa (%)
70
60
50
40
30
20
10
0
*
*
**
**
***
Rec-BSPH1 (µg/mL) 0 5 10 10 30 30 60
Rec-BSPH2 (µg/mL) 0 5 0 10 0 30 0

Article 4

Murine Binder of SPerm Protein Homolog 1 (BSPH1): New Player in HDL-Induced Capacitation.

Geneviève Plante et Puttaswamy Manjunath. In press, *Reproduction* (Janvier 2015)

Résumé

Les protéines Binder of SPerm (BSP) sont ubiquitaires chez les mammifères et sont exclusivement exprimées dans le tractus génital mâle. La fonction primaire associée aux protéines BSP découle de leur propriété de promouvoir la capacitation des spermatozoïdes. Chez la souris, deux protéines (BSPH1 et BSPH2) ont été étudiées. En utilisant des protéines recombinantes, il a été démontré que BSPH1 pouvait se lier à la membrane des spermatozoïdes épididymaires et promouvoir la capacitation des spermatozoïdes in vitro. Le but de la présente étude était d'évaluer le rôle de la protéine de souris BSPH1 endogène dans la capacitation induite par l'albumine de sérum bovin (BSA) et par les lipoprotéines de haute densité (HDL). L'effet d'anticorps, de fragments d'IgG monovalents liant l'antigène (Fab) et de fragments d'IgG divalents liant l'antigène ($F(ab')_2$) spécifiques pour la protéine BSPH1 sur la capacitation induite par la BSA et les HDL a donc été testé. Les résultats ont démontré que BSPH1 n'a pas de rôle direct au niveau de la capacitation induite par la BSA. Toutefois, les anticorps, les Fabs et les $F(ab')_2$ étaient capables de bloquer la capacitation induite par les HDL et d'inhiber l'augmentation de la phosphorylation des résidus tyrosine induite par les HDL suggérant une interaction spécifique entre BSPH1 et les HDL. Les résultats obtenus suggèrent également que la protéine de souris BSPH1 pourrait être un nouveau facteur important pour la capacitation induite par les HDL. Puisque la protéine BSPH1 de souris est un orthologue de la protéine humaine BSPH1, cette étude pourrait mener à une meilleure compréhension de la fonction et de l'importance de la protéine humaine dans la fertilité masculine.

Contribution

Pour cet article, j'ai contribué au design expérimental, à l'acquisition des données et à l'analyse des données pour toutes les expériences. J'ai également rédigé l'article.

Murine Binder of SPerm Protein Homolog 1 (BSPH1): New Player in HDL-Induced Capacitation.[1]

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Short title: Murine BSPH1 and HDL-induced capacitation

ABSTRACT

Binder of SPerm (BSP) proteins are ubiquitous amongst mammals and are exclusively expressed in male genital tract. The main function associated with BSP proteins is their ability to promote sperm capacitation. In mice, two proteins (BSPH1 and BSPH2) have been studied. Using recombinant strategies, BSPH1 was found to bind to epididymal sperm membranes and promote sperm capacitation *in vitro*. The goal of the present study was to evaluate the role of native murine BSPH1 protein in sperm capacitation induced by bovine serum albumin (BSA) and High-Density Lipoproteins (HDL). The effect of antibodies, antigen-binding fragments (Fabs) and $F(ab')_2$ specific against murine BSPH1 on BSA- and HDL-induced capacitation was tested. Results show that BSPH1 has no direct role in BSA-induced capacitation. However, antibodies, Fabs and $F(ab')_2$ could block capacitation induced by HDL and could inhibit the HDL-induced increase in tyrosine phosphorylation, suggesting a specific interaction between HDL and BSPH1. Results suggest that murine BSPH1 proteins in mice could be a new important piece of the puzzle in sperm capacitation induced by HDL. Since murine BSPH1 is orthologous to human BSPH1, this study could also lead to new insights on the functions and the importance of the human protein in male fertility.

INTRODUCTION

In mammals, for fertilization to occur, sperm have to go through several maturation steps. One of the key maturation steps taking place in the female genital tract is called capacitation. It is a prerequisite for sperm to undergo acrosome reaction, recognize an oocyte, interact with it and then fertilize it. Although capacitation was first described over 60 years ago, this process is still not fully understood. Capacitation is usually associated with an increase in intracellular pH, an increase in calcium permeability as well as changes in the lipid composition of the sperm plasma membrane, including a decrease in the cholesterol/phospholipid ratio (Go & Wolf 1983, Langlais & Roberts 1985, Suarez 1996, de Lamirande *et al.* 1997, Visconti & Kopf 1998). It is also accompanied by the time-dependent activation of many different signaling pathways including the protein kinase A (PKA) pathway, the protein kinase C (PKC) pathway, the extracellular signal-regulated kinase (ERK) pathway and the phosphatidyl-inositol-3-kinase (PI3K)/Akt pathway leading to an increase in the level of protein tyrosine phosphorylation (Breitbart *et al.* 1992, Visconti *et al.* 1995b, de Lamirande *et al.* 1997, Fisher *et al.* 1998, Luconi *et al.* 1998, de Lamirande & Gagnon 2002, Nauc *et al.* 2004).

Capacitation can be induced *in vitro* by incubating sperm in defined media. To induce capacitation, these media require some essential components including calcium, bicarbonate as well as sterol acceptors. In mice, the most commonly used sterol acceptors are bovine serum albumin (BSA) and high-density lipoproteins (HDL) (Visconti *et al.* 1999b, Xia & Ren 2009). Some proteins from the male genital tract have been shown to be important for sperm capacitation by preventing a premature capacitation and/or by promoting sperm capacitation. Proteins from the Binder of SPerm (BSP) superfamily (called Bovine Seminal Plasma proteins

prior to the new nomenclature (Manjunath *et al.* 2009)) have been shown to play a role in both prevention and promotion of capacitation. They were first identified in the bovine seminal plasma where BSP1, BSP3 and BSP5 (previously called PDC-109 or BSP-A1/A2, BSP-A3 and BSP-30K, respectively) represent approximately 60% of the total seminal plasma proteins (Manjunath 1984, Manjunath *et al.* 1987, Seidah *et al.* 1987). These three proteins are secreted by the seminal vesicles, can bind to sperm via an interaction with choline phospholipids (Desnoyers & Manjunath 1992). They have been identified as important players in the glycosaminoglycans (GAG)-induced and HDL-induced capacitation in bovine (Thérien *et al.* 1995, Thérien *et al.* 1997, Therien *et al.* 2005).

Other members of the BSP superfamily have been identified in species such as boar, ram, goat, stallion and bison and more recently in human and in mice (Calvete *et al.* 1995, Calvete *et al.* 1997, Menard *et al.* 2003, Villemure *et al.* 2003, Boisvert *et al.* 2004, Bergeron *et al.* 2005, Fan *et al.* 2006). The BSP proteins are all structurally similar as they are composed of a variable N-terminal domain followed by two fibronectin type-II (Fn2) domains arranged in tandem (Manjunath *et al.* 2009). However, the two BSP homologs in mouse (BSPH1 and BSPH2) and the BSP homolog in human (BSPH1) are slightly different from their other counterparts as they are expressed exclusively in the epididymis and represent only a small quantity of the total seminal plasma proteins (Lefebvre *et al.* 2007). Despite those differences, recent studies using recombinant proteins have shown that all three proteins share many biochemical characteristics with the BSP proteins expressed by the seminal vesicles including binding to gelatin, GAGs and sperm membrane (Plante *et al.* 2012, Plante *et al.* 2014a, Plante *et al.* 2014b). These studies also demonstrated that incubation of sperm with an

excess of murine or human rec-BSPH1 but not rec-BSPH2 was sufficient to induce sperm capacitation (Plante *et al.* 2012, Plante *et al.* 2014a, Plante *et al.* 2014b).

Although our previous studies on murine and human BSP seemed to indicate a possible role of considerable importance in capacitation, studies were performed using an excess of proteins and as such could not reflect entirely *in vivo* conditions. However, some results demonstrated that incubation of murine sperm with antibodies specific against BSPH1 to block the native protein caused a dose-dependent inhibition of BSA-induced capacitation (Plante *et al.* 2012). In the current study, we attempted to get further insight on the molecular mechanism involving murine BSPH1 in capacitation induced by BSA and by HDL.

MATERIALS AND METHOD

Animals

Pathogen-free CD-1 outbred mice were purchased from Charles River Laboratories (Kingston, NY, USA) and were kept in the animal care facility of the research center. Animals were given ad libitum food and filtered tap water, and maintained under a 14:10-h light-dark cycle. Studies were approved by the Maisonneuve-Rosemont Hospital Ethics Committee (Protocol #2014-10) and mice were treated according to the guidelines of the Canadian Council of Animal Care.

Preparation of fragment antigen-binding (Fab) and F(ab')$_2$ fragment

Affinity-purified antibodies raised against $(His)_6$-tagged recombinant BSPH1 (anti-BSPH1) were prepared as previously described (Plante *et al.* 2012). Following purification by affinity chromatography on a Protein-A-sepharose column and a rec-BSPH1-Affi-gel 15 column, anti-BSPH1 antibodies were concentrated (~1 mg/ml). Fab fragments were prepared

using these antibodies (Kontou *et al.* 1996, Lane *et al.* 1999). Briefly, papain from papaya latex (Sigma–Aldrich, Oakville, ON, Canada) was activated for 15 min at 37°C in digestion buffer (phosphate buffered saline (PBS) containing 10 mM EDTA and 2 mM L-Cystein). The enzyme was added to the antibodies at a 1:15 (papain: antibody) ratio. The reaction was incubated at 37°C for 7 hours. To stop the reaction, the digestion mix was incubated at 4°C overnight with 25 mM iodoacetamine. The digestion product was then dialyzed against PBS (pH 8.0) and purified on a Protein-A-sepharose column. Unbound material containing the Fab was washed from the column with 10 volumes of PBS and concentrated (~1 mg/ml).

$F(ab')_2$ were prepared using Pierce $F(ab')_2$ preparation kit (Pierce, Rockford, IL, USA) as described by the manufacturer. Control Fab (Fab-IgG) and $F(ab')_2$ fragment (F2-IgG) were prepared similarly using IgGs purified from pre-immune normal rabbit serum (IgG).

Preparation of High-Density lipoproteins (HDL)

HDL were isolated from human serum by density gradient ultracentrifugation as previously described (Thérien *et al.* 1997). They were dialyzed against buffer containing 0.9% NaCl, 1 mM EDTA, 25 mM Hepes pH 7.4. HDL were subsequently kept under nitrogen at 4°C. The purity of the lipoproteins was verified by agarose gel electrophoresis using the Paragon lipoprotein (Lipo) electrophoresis kit (Beckman Instruments, Fullerton, CA) and following the protocol described by the manufacturer. The concentration of lipoproteins was measured according to the modified Lowry procedure (Markwell *et al.* 1978).

Preparation of sperm

Male mice (10-18 weeks old) were sacrificed by cervical dislocation. Cauda epididymides were removed and placed in warm PBS. Epididymides were then cleaned to remove fat and blood. They were then cut four to six times with scissors and placed in 1 ml of pre-warmed modified Krebs-Ringer medium (Whitten's HEPES; WH; 100 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.5 mM D-glucose, 1 mM sodium pyruvate, 4.8 mM L(+)-lactic acid hemicalcium salt in 20 mM Hepes) buffered medium, pH 7.4 (osmolality 315 mOsm/kg) and placed at 37°C for 10 min. Following incubation, epididymal debris were removed and sperm were resuspended by gentle swirling (Moore *et al.* 1994). For each experiment, sperm from three to four mice were pooled and used for the different assays.

Inhibition of capacitation assay

For this set of experiments, sperm were collected as described above, washed once with 5 ml of WH medium (10 min at 200 × *g*) and resuspended in 500 µl of WH medium. For BSA-induced capacitation studies, 2×10^6 washed sperm were then incubated 1 h at 37°C in 1 ml WH medium containing 5 mg/ml of BSA alone or in 1 ml WH medium containing BSA and different concentrations of Fab, $F(ab')_2$ or anti-BSPH1. As negative controls, sperm were also incubated under non-capacitating conditions in WH medium depleted of bicarbonate and BSA, with BSA and 5.6 µg/ml of Fab-IgG or with BSA and 5.6 µg/ml of F2-IgG. For HDL-induced capacitation, different concentrations of HDL were tested to determine optimal conditions. Then, same conditions as the ones used for BSA-induced capacitation were tested, but instead of BSA, 25 µg HDL were used.

Following the incubation, 200 µl of the sperm suspension were incubated an additional 30 min at 37°C with or without 5 µM of calcium ionophore A23187 (Sigma-Aldrich). Sperm were then fixed with 200 µl of 8% paraformaldehyde for 30 minutes at room temperature, centrifuged for 2 min at 8000 × *g* and washed 2 times with 0.1 M ammonium acetate (pH 9.0). They were finally resuspended in a final volume of 100 µl of the same solution. 20 µl of this suspension were smeared on microscopic slides. Slides were dried and stained using the Coomassie Brilliant Blue staining technique (Moller *et al.* 1990). Slides were placed successively in water, methanol and water for 5 minutes each, stained 2 minutes in a solution of 0.22 % Coomassie Blue G-250 in 50% methanol and 10% acetic acid solution and washed with water. Acrosomal status of 400 sperm was assessed for each condition.

Tyrosine phosphorylation

Tyrosine phosphorylation experiments were performed as previously described (Visconti *et al.* 1995a). 1×10^6 sperm collected as described above were incubated for 90 minutes at 37°C in 500 µl WH medium containing 12.5 µg/ml of HDL alone or with 12.5 µg/ml of HDL in the presence of different concentrations of Fab or 11.4 µg/ml of Fab-IgG. Following incubation, sperm were pelleted by 1 min centrifugation at 16 000 × *g*. Sperm pellet was washed with 1 ml of PBS containing 1 mM orthovanadate and centrifuged another minute. Pellet was resuspended in 25 µl of Laemmli sample buffer without mercaptoethanol and boiled 5 minutes (Laemmli 1970). After one last centrifugation at 16 000 × *g* for 1 min, the supernatant was transferred to a new tube, boiled in the presence of 5% β-mercaptoethanol for 5 minutes, and then subjected to SDS-PAGE (10% gels).

Isolation of light buoyant-density detergent resistant membrane (DRM) fractions

Isolation of DRM was done as previously described (Sleight *et al.* 2005). A total of 60 million sperm isolated from cauda epididymides were washed twice with 1 ml of WH media and resuspended in 400 µl of TEN buffer (25 mM Tris-HCl (pH 7.3), 150 mM NaCl and 5 mM EDTA) containing 0.5% Triton X-100, Complete Mini, EDTA-free protease inhibitor tablet (1 tablet/10 ml; Roche, Mannheim, Germany) and 1 mM of PMSF. The pellet was Dounce homogenized (30 strokes). It was then sonicated 1 second at 50% five times and kept on ice 1 min between each burst. Cell lysate was then rotated at 4°C for 45 min. To separate the DRM according to their density, sperm lysate was adjusted to 40% sucrose with the addition of 400 µl 80% sucrose in TEN buffer and placed at the bottom a of 2-ml centrifuge tube. This solution was gently overlaid with 800 µl of 30% sucrose in TEN buffer and 400 µl of 5% sucrose in TEN. The sucrose gradient was then centrifuged at 100 000 × *g* for 18 h. Fractions of 200 µl were collected from the top to the bottom of the gradient. The pellet at the bottom of the tube was resuspended in 200 µl of 10 mM Tris-HCl pH 7.4 and kept for the analysis. 50 µl of 5X sample buffer was added to each fraction. Fractions were finally boiled for 10 minutes and 1/10th of each fraction was analyzed on 15% polyacrylamide gels.

Protein electrophoresis and western blotting

SDS–PAGE was performed according to the method of Laemmli (Laemmli 1970) using the Mini-Protean 3 apparatus from Bio-Rad (Mississauga, ON, Canada). Gels were transferred electrophoretically to Immobilon-P PVDF membranes (Millipore, Nepean, ON, Canada). For the tyrosine phosphorylation assay, immunodetection was performed at room temperature for 1 hour with a monoclonal antibody against phosphotyrosine (clone 4G10;

Millipore) using a dilution of 1:10 000. For analysis of the DRM fractions, membranes were incubated overnight at 4°C with rabbit polyclonal antibodies against Caveolin-1 (clone N-20; Santa Cruz; dilution 1:500) or rec-BSPH1 purified on Protein-A-sepharose column (dilution 1:1000). The bands were revealed using chemiluminescence reagent (Perkin–Elmer, Boston, MA, USA) and a Fuji LAS-3000 image analyzer (Fujifilm; Stamford, CT, USA). To verify protein loading, membranes were stained with a solution of 0.5% Amido Black 10B (Bio-Rad).

Statistical Analysis

Data are presented as the mean ± S.E.M. Differences were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test using GraphPad Prism 5 (version 5.03).

RESULTS

BSA-induced capacitation

Previous experiments using anti-BSPH1 antibodies demonstrated that they could cause dose-dependent inhibition of BSA-induced capacitation (Plante *et al.* 2012). To investigate this inhibition further, similar experiments were performed using Fab and $F(ab')_2$ fragments. Since Fab and $F(ab')_2$ accounts for approximately two-thirds of an IgG, concentrations used in these experiments were two-thirds of the concentration of anti-BSPH1 previously used (Plante *et al.* 2012). In general, only capacitated sperm can undergo the acrosome reaction (AR). Capacitation was therefore assessed by the ability of sperm to undergo AR induced by calcium ionophore A23187. As seen on Figure 1, under non-capacitating conditions, the basal level of

AR in the absence of A23187 was 18 ± 2 %. This level was unaffected by the addition of A23187 under non-capacitating conditions and was constant under all conditions tested in the absence of A23187 (grey bars). Following the addition of 5 mg/ml BSA, in the presence of A23187, the level of AR increased to 73 ± 1%. As shown previously (Plante *et al.* 2012), in the presence of 8.4 μg/ml anti-BSPH1, percentage of AR sperm was comparable to basal level. Addition Fab did not affect the level of AR at any of the concentrations tested (Fig 1A). However, incubation with $F(ab')_2$ caused a significant decrease in the level of AR at concentrations as low as 0.3 μg/ml (Fig 1B). Control using Fab-IgG and F2-IgG produced using IgG from pre-immune normal rabbit serum did not alter the levels of AR.

To verify that the effect observed on capacitation and AR was not due to the death of sperm, sperm viability was assessed based on sperm motility. In the beginning of the experiments, 90-95% sperm were motile. Following incubation, prior to the addition of A23187, motility decreased slightly (5-10%), but this decrease was the same for all conditions tested. This was also observed for HDL-induced capacitation. Results are based on percentages of all cells (motile and immotile).

HDL-induced capacitation

Articles previously published using HDL to induce murine sperm capacitation, assessed the capacitated state of sperm by tyrosine phosphorylation and used high concentrations of sperm during incubation (Visconti *et al.* 1999a). Therefore, prior to testing the effect of anti-BSPH1 antibodies on HDL-induced capacitation, several concentrations of HDL were tested to determine the appropriate conditions necessary to induce capacitation in murine sperm using 2×10^6 sperm/ml and assess capacitated state by the ability of sperm to undergo AR

(Figure 2). Following incubation with A23187, under non-capacitating conditions, the level of AR was of 25 ± 1%. Addition of 5 µg/ml of HDL did not cause a significant increase in AR level but the addition of 25 µg/ml of HDL increased it to 54 ± 1%. Similar percentages of AR sperm were obtained with higher concentrations of HDL. Therefore, 25 µg/ml of HDL were used for the inhibition studies.

In the presence of anti-BSPH1 antibodies, results obtained for HDL-induced capacitation were similar to the ones obtained for BSA-induced capacitation (Fig 3A). The addition of 0.5 µg/ml anti-BSPH1 decreased level of AR significantly and the addition of 8.4 µg/ml anti-BSPH1 dropped the percentage of AR back to the basal level. However, different results were obtained when sperm were incubated with HDL in the presence of Fab (Fig 3B) as the addition of the antibody fragments caused dose-dependent decrease in the level of AR. Decrease in the percentages of AR sperm were significant following the addition of 1.4 µg/ml of antibody fragments. Identical results were obtained when sperm were incubated with $F(ab')_2$ fragments (Fig 3C). Addition of IgG, Fab-IgG of F2-IgG had no effect on sperm AR.

Tyrosine phosphorylation

HDL-induced capacitation has been shown to increase the phosphorylation of tyrosine residues in signaling proteins. Therefore, the effect of Fab on the phosphorylation induced during incubation of sperm with HDL was tested. As seen on Figure 4A, at the beginning of the incubation or following 90 minutes incubation without HDL, levels of phosphorylation were very low. Addition of 12.5 µg/ml increased the phosphorylation of sperm proteins, which was inhibited by the addition of 11.4 µg/ml of Fab. Lower concentrations of antibody fragments had no effect. 11.4 µg/ml of Fab-IgG caused a slight decrease in the

phosphorylation, but not as significant as the one observed for specific BSPH1 Fab. A decrease in phosphorylation was also observed when sperm were incubated with 25 μg/ml of HDL, but the effect was not as pronounced (not shown). Equal loading of proteins on acrylamide gel was verified by staining the membrane with Amido Black (Fig. 4B). Amount of protein loaded for sperm incubated in the absence of HDL was lower than the amount of proteins for sperm incubated with HDL. However, the difference in proteins loaded is not sufficient to explain the absence of tyrosine phosphorylation in those two samples. No difference in loading was observed for sperm incubated with HDL in the presence of different concentrations of Fab and antibodies.

DRM fractions

The sperm plasma membrane contains domains called lipid rafts that are highly enriched in cholesterol and sphingolipids. These domains are known to be insoluble in detergent at 4°C and possess light buoyant density on sucrose density gradient. Isolation of DRM has often been used to identify raft (Thaler *et al.* 2006, Miranda *et al.* 2009). In order to determine if murine BSPH1 is located in those domains, DRM fractions of epididymal sperm membranes were separated on sucrose density gradient and different fractions were analyzed by Western blot (Figure 5). In the sperm membrane, a fraction of the Caveolin 1 (CAV1) protein is found in lipid rafts. As such, CAV1 was found in the light fractions of the density gradient. Following Western blot, CAV1 was observed in fractions 4 to 10 and in the pellet containing cell debris. As oppose to this positive control, BSPH1 was not found in the light fractions and was observed solely in the pellet indicating that it is not found in the lipid rafts.

DISCUSSION

BSA-induced capacitation

BSA is often used to induce capacitation *in vitro*. It acts as a sterol acceptor and removes cholesterol from sperm membranes. This causes a destabilization of the membrane and renders it more fluid, leading to tyrosine phosphorylation and calcium intake (Salicioni *et al.* 2007). Our previous studies demonstrated that co-incubation of sperm with BSA and anti-BSPH1 antibodies could inhibit completely the capacitation induced by BSA (Plante *et al.* 2012). Three molecular explanations were raised to justify this inhibition. The first explanation was that the big size of the antibodies bound to native BSPH1 on sperm membrane blocked BSA's access to the membrane cholesterol (Fig 6A). The second theory was that antibodies were preventing a direct interaction between BSA and BSPH1 (Fig 6B). The last explanation derived from the ability of antibodies to bind to two different antigens. Bovine BSP proteins are known to coat the sperm surface, prevent free movement of the phospholipids and stabilize sperm membrane (Muller *et al.* 1998, Manjunath & Thérien 2002). Therefore, in a similar manner, binding of antibodies to the native BSPH1 bound to the sperm surface could have created a stable network between BSPH1 proteins, thereby stabilizing the sperm membrane and preventing BSA-induced capacitation (Fig 6C). The third hypothesis at the time was favoured, but in the current study, experiments were designed to confirm it.

$F(ab')_2$ and Fab fragments are digested products of antibodies. $F(ab')_2$ are less bulky than antibodies as they do not possess the Fc fragment but they can still bind two antigens. Fab fragments on the contrary are only composed of one antigen-binding domain. Repeating the inhibition experiments using these tools had three possible outcomes. The first hypothesis would be confirmed if only anti-BSPH1 antibodies could inhibit BSA-induced capacitation.

The second hypothesis would be confirmed if Fab, $F(ab')_2$ and antibodies could inhibit capacitation and the third hypothesis would be verified if antibodies and $F(ab')_2$, but not Fab could cause inhibition. Results obtained show that the third hypothesis is the most probable one to explain the inhibition of BSA-induced capacitation by anti-BSPH1 antibodies. They also show that BSPH1 is most probably not directly involved in capacitation induced by BSA. Furthermore, these results suggest that BSPH1 could be implicated in the stabilization of the sperm membrane to prevent premature capacitation, as is the case for bovine BSP proteins.

HDL-induced capacitation

HDL is a macromolecule composed of phospholipids, triglycerides, cholesterol and apoproteins. Its major protein is apolipoprotein A-I (apoA-I). It is a major factor implicated in cholesterol transport and, as such, has also been shown to induce capacitation. Just like BSA, HDL can induce *in vitro* capacitation by creating cholesterol and phospholipids efflux from sperm membrane (Therien *et al.* 1998). In many species, HDL is found in the follicular and oviductal fluid making it an ideal candidate to promote capacitation *in vivo* (Travis & Kopf 2002). Previous studies reported that to induce capacitation-associated tyrosine phosphorylation in 1×10^7 sperm, a concentration of 300 µg/ml of HDL were necessary (Visconti *et al.* 1999a). In the current study, under the conditions used (1×10^6 sperm), a minimum of 12.5 µg/ml of HDL was necessary to induce tyrosine phosphorylation and 25 µg/ml of HDL were necessary to see an increase in the level of AR following incubation with A23187.

In bovine, it has been shown that BSP1, BSP3 and BSP5 are all able to bind HDL and more specifically to apoA-I (Manjunath *et al.* 1989). HDL also contains choline

phospholipids, which could also be responsible for the BSP binding. Through this interaction, bovine BSP proteins in bovine can increase the stimulation of capacitation caused by HDL (Thérien *et al.* 2001). To determine if murine BSPH1 could be implicated in a similar mechanism, the effect of anti-BSPH1 on the HDL-induced capacitation in murine sperm was tested. Interestingly, as opposed to what was observed during BSA-induced capacitation, Fab alone were able to decrease capacitation to a level comparable to the inhibition caused by the full antibodies. This result suggested a more direct role of BSPH1 in the HDL-induced capacitation process. Since BSP proteins are known to interact directly with HDL and no direct interaction with other sperm protein has ever been reported for members of the BSP superfamily, results suggest that a direct interaction between HDL and BSPH1 could be necessary for capacitation.

Tyrosine phosphorylation

To confirm the inhibition effect of anti-BSPH1 on HDL-induced capacitation, effect of Fab on tyrosine phosphorylation was tested. As was observed before, lower concentrations of sterol acceptors were necessary to detect an increase in tyrosine phosphorylation when compared to concentrations needed to induce AR (Visconti *et al.* 1995a). The addition of 11.4 µg/ml of Fab decreased the tyrosine phosphorylation back to what was observed without the addition of HDL. Previous studies in human and in bovine showed that BSP proteins do not seem to be directly implicated in the phosphorylation cascade (Lane *et al.* 1999, Plante *et al.* 2014b). In mice, it is believed that sterol acceptors such as HDL or BSA change the fluidity of the membrane by removing cholesterol and phospholipids. The changes in fluidity lead to an increase in the uptake of calcium and bicarbonate stimulating the production of cyclic AMP

(cAMP). This increase then causes the activation of the PKA pathway and subsequently, to the increase in tyrosine phosphorylation (Visconti *et al.* 1999a). Based on this theory, it is possible that as observed in bovine, BSPH1 could help the transfer of cholesterol and phospholipids to the HDL. However, in the murine model, there is no evidence at the moment showing that BSP proteins would be loaded on HDL to help remove phospholipids and cholesterol. Experiments performed using recombinant proteins rather suggest that the proteins remain on the surface of the sperm, change location on the surface of the head and could be implicated in other steps of fertilization such as sperm-egg interaction.

Lipid rafts

Sperm membranes contain domains that are enriched in cholesterol, gangliosides and sphingolipids (Sleight *et al.* 2005). These domains called lipid rafts also contain many proteins implicated in the regulation of cell signaling, AR and cumulus- and/or zona pellucida-binding processes (Bou Khalil *et al.* 2006, Thaler *et al.* 2006). Many of the proteins found in lipid rafts are acquired during epididymal maturation (Gadella *et al.* 2008). Furthermore, recent studies showed that the removal of cholesterol by BSA and apoA-I as well as the removal of phospholipids by apoA-I during sperm capacitation occurs from non-raft fractions (Mendez *et al.* 2001, Boerke *et al.* 2013). Most proteins implicated in the cholesterol/phospholipid removal were also located in non-raft fractions. Results obtained in the current study also placed BSPH1 in non-raft fraction, suggesting once again a role for BSPH1 in the cholesterol/phospholipid removal, which occurs during capacitation.

Based on our studies of murine BSPH1, we propose the following mechanism of sperm capacitation (Fig 7). During epididymal maturation, sperm enter in contact with BSPH1,

which bind to the sperm surface via an interaction with choline phospholipids. This interaction stabilizes the membrane and prevents premature capacitation and AR. Following ejaculation, sperm enter the oviduct where they encounter HDL. Via a specific interaction, BSPH1 transfer phospholipids and cholesterol to HDL particles, that destabilizes the membrane. This causes the activation of the PKA and subsequent signalling pathways leading to capacitation.

As it is the case in many cellular processes, alternative pathways and other proteins including ATP-binding cassette transporters and the scavenger receptor SR-BI have been shown to be implicated in HDL-induced capacitation as well (Travis & Kopf 2002, Morales *et al.* 2008). However, the fact that Fab specific for BSPH1 were able to inhibit completely HDL-induced capacitation suggest a crucial role in BSPH1 in murine sperm capacitation. In mice, a decrease in the sperm cholesterol content has also been noted during epididymal maturation, which has been associated with the presence of ApoA-I and ApoJ in the epididymis (Rejraji *et al.* 2006, Morales *et al.* 2008). BSPH1 being expressed in the caput epididymis could therefore be also implicated in the plasma membrane changes taking place during epididymal maturation (Lefebvre *et al.* 2007). Ongoing studies of BSP knock-out mice should soon bring answers to those questions.

In conclusion, results of this study show that like bovine BSP proteins, murine BSPH1 is implicated in the HDL-induced capacitation and could be a key player in this process. Since murine BSPH1 shares a high level of sequence identity with the human BSPH1 it is highly possible that this mechanism would also apply in human fertility processes.

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The authors have no conflict(s) of interest to declare.

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FIGURE LEGENDS

Fig 1. *Effect of anti-BSPH1 Fab and F(ab')$_2$ on BSA-induced capacitation in murine sperm.* Capacitation was assessed by the ability of sperm to undergo the AR induced by A23187 ionophore. Epididymal sperm were incubated for 60 min with or without 5 mg/ml of BSA, in the presence of different concentrations of Fab, F(ab')$_2$ or anti-BSPH1. As controls, sperm were incubated with 5.6 µg/ml of Fab-IgG or 5.6 µg/ml of F2-IgG. Sperm were then incubated with (black bars) or without (grey bars) calcium ionophore A23187 for 30 min. Sperm were smeared on slides and analyzed by Coomassie Blue staining. (A) Effect of Fab on sperm capacitation. (B) Effect of F(ab')$_2$ on sperm capacitation. A minimum of 400 sperm per condition were evaluated. Data are means ± S.E.M. of three independent experiments. *: significant difference compared to BSA alone (control, $P < 0.001$).

Fig 2. *Effect of HDL on capacitation in murine sperm.* Capacitation was assessed by the ability of sperm to undergo the AR induced by A23187 ionophore. Epididymal sperm were incubated for 60 min alone, with different concentrations of HDL or with 5 mg/ml of BSA as positive control. They were then incubated with (black bars) or without (grey bars) calcium ionophore A23187 for 30 min. Sperm were smeared on slides and analyzed by Coomassie Blue staining. A minimum of 400 sperm per conditions were evaluated. Data are means ± S.E.M. of four independent experiments. *: significant difference compared to BSA alone (control, $P < 0.001$).

Fig 3. *Effect of anti-BSPH1, Fab and F(ab')$_2$ fragments on murine sperm capacitation induced by HDL.* Capacitation was assessed by the ability of sperm to undergo the AR induced by A23187 ionophore. Epididymal sperm were incubated for 60 minutes alone or with 25 µg/ml of HDL in the presence of different concentrations of anti-BSPH1, Fab or F(ab')$_2$ fragments. As controls, sperm were incubated in the presence of 5.6 µg/ml of Fabs-IgG, 5.6 µg/ml of F2-IgG or 8.4 µg/ml of IgG. Sperm were then incubated for 30 minutes with (black bars) or without (grey bars) of calcium ionophore A23187. Sperm were smeared on slides and analyzed by Coomassie Blue staining. (A) Effect of anti-BSPH1 antibodies. (B) Effect of Fabs on sperm capacitation. (C) Effect of F(ab')$_2$ on sperm capacitation. A minimum of 400 sperm per conditions were evaluated. Data are means ± S.E.M. of four independent experiments. *: significant difference compared to HDL Alone (control, $P < 0.001$).

Fig 4. *Effect of Fab on tyrosine phosphorylation during HDL-induced capacitation.* Epididymal sperm were incubated for 90 minutes alone or with 12.5 µg/ml of HDL in the

presence of different concentrations Fab or 11.4 µg/ml of Fabs-IgG. (A) Proteins were separated by SDS-PAGE on 10% acrylamide gel, transferred on a PVDF membrane and probed with anti-phosphotyrosine antibody. (B) Equal loading of proteins on acrylamide gel was verified by staining the membrane with Amido Black.

Fig 5. *Identification of murine BSPH1 in the non-raft fraction of the sperm plasma membrane.* Epididymal sperm were lysed in TEN buffer containing 0.5% Triton X-100 and DRM lipid rafts were isolated by sucrose gradient. Following centrifugation, 200 µl fractions were collected from top to bottom and the pellet was resuspended in 200 µl of Tris-HCl. $1/10^{th}$ of each fraction was separated on 15% acrylamide gel transferred on a PVDF membrane and probed with anti-CAV1 or anti-BSPH1 antibodies. This experiment was repeated four times with similar results.

Fig 6. *Proposed models of inhibition of BSA-induced capacitation by anti-BSPH1.* **(**A) Due to big size of the antibodies bound to native BSPH1 on sperm membrane, BSA is unable to access to the membrane cholesterol. (B) Antibodies bind native BSPH1 on sperm membrane and prevent a direct interaction between BSA and BSPH1 necessary for capacitation. (C) Antibodies bind to the native BSPH1 bound to sperm and create a stable network between BSPH1 proteins, stabilizing the sperm membrane and preventing BSA-induced capacitation.

Fig 7. *Proposed mechanism of sperm capacitation by BSPH1.* During epididymal maturation, sperm enter in contact with BSPH1, which bind to the sperm surface via an

interaction with choline phospholipids. This interaction stabilizes the membrane and prevents premature capacitation and AR. Following ejaculation, sperm enter the oviduct where they encounter HDL. Via a specific interaction, BSPH1 transfer phospholipids and cholesterol to HDL particles, causing a decrease in the cholesterol/phospholipid ratio that destabilizes the membrane. This causes an increase in intracellular pH, calcium and cAMP that causes increase in protein tyrosine phosphorylation and subsequently capacitation.

FIGURE 1

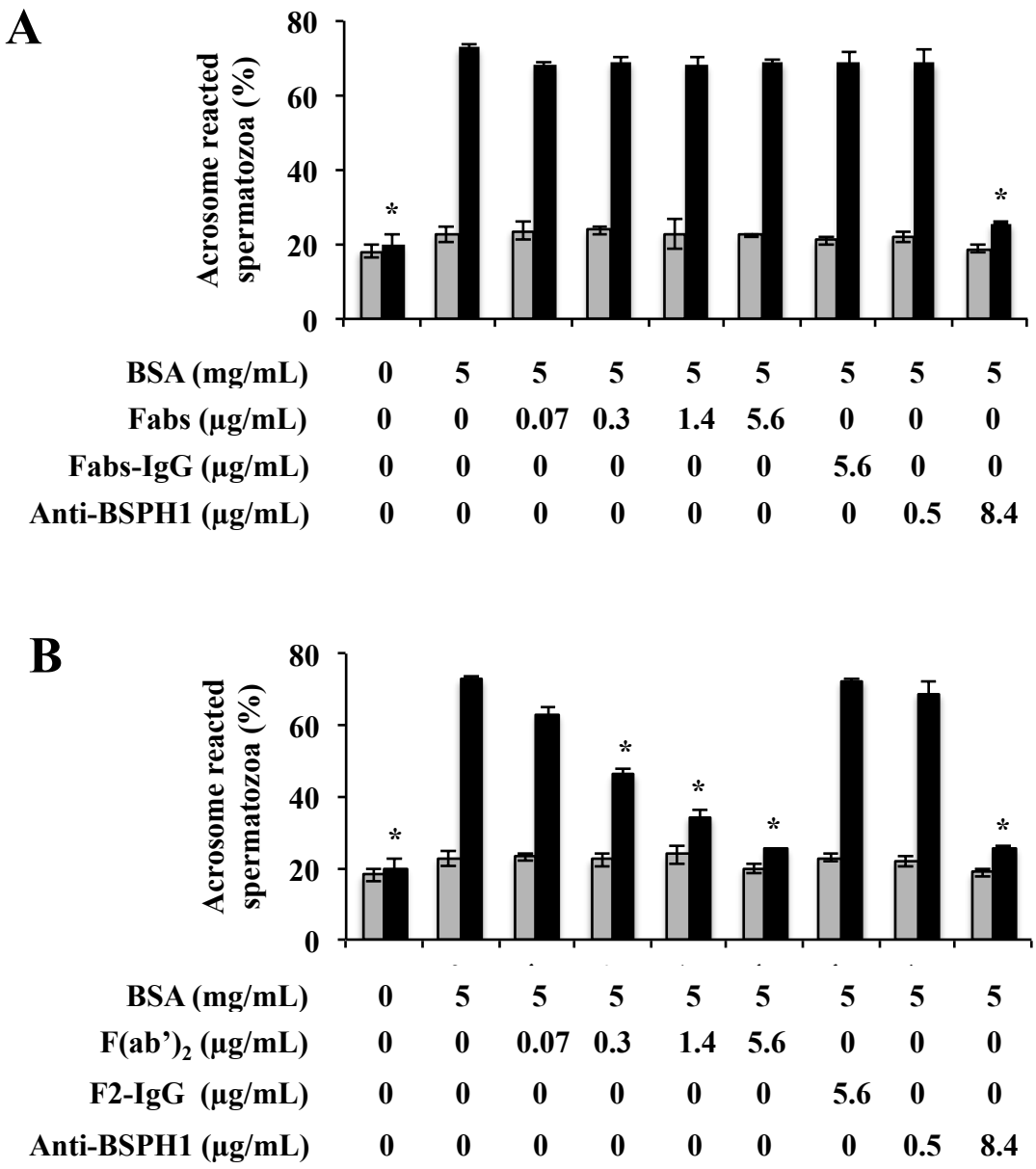

A
Acrosome reacted spermatozoa (%)
80
60
40
20
0
*
*
BSA (mg/mL) 0 5 5 5 5 5 5 5 5
Fabs (µg/mL) 0 0 0.07 0.3 1.4 5.6 0 0 0
Fabs-IgG (µg/mL) 0 0 0 0 0 0 5.6 0 0
Anti-BSPH1 (µg/mL) 0 0 0 0 0 0 0 0.5 8.4
B
Acrosome reacted spermatozoa (%)
80
60
40
20
0
*
*
*
*
*
BSA (mg/mL) 0 5 5 5 5 5 5 5 5
F(ab')2 (µg/mL) 0 0 0.07 0.3 1.4 5.6 0 0 0
F2-IgG (µg/mL) 0 0 0 0 0 0 5.6 0 0
Anti-BSPH1 (µg/mL) 0 0 0 0 0 0 0 0.5 8.4

FIGURE 2

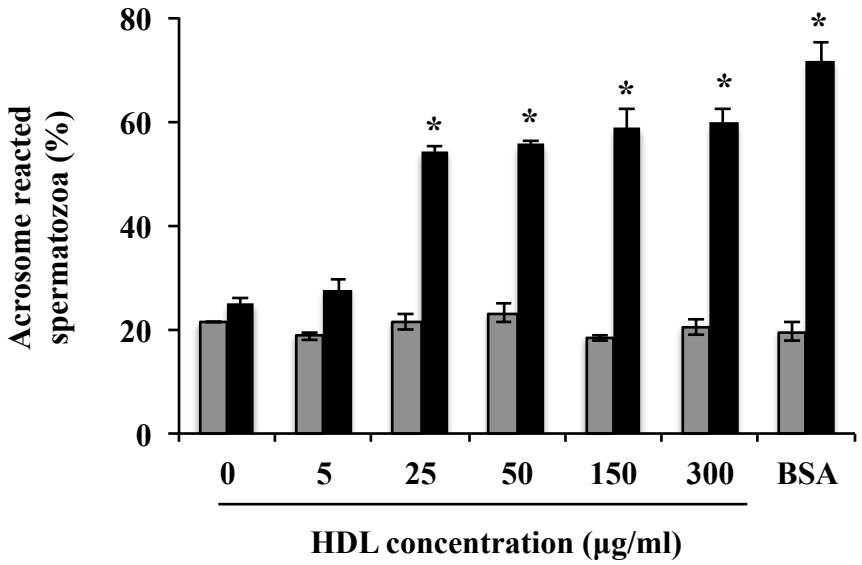

Acrosome reacted spermatozoa (%)
80
60
40
20
0
*
*
*
*
*
0
5
25
50
150
300
BSA
HDL concentration (µg/ml)

FIGURE 3

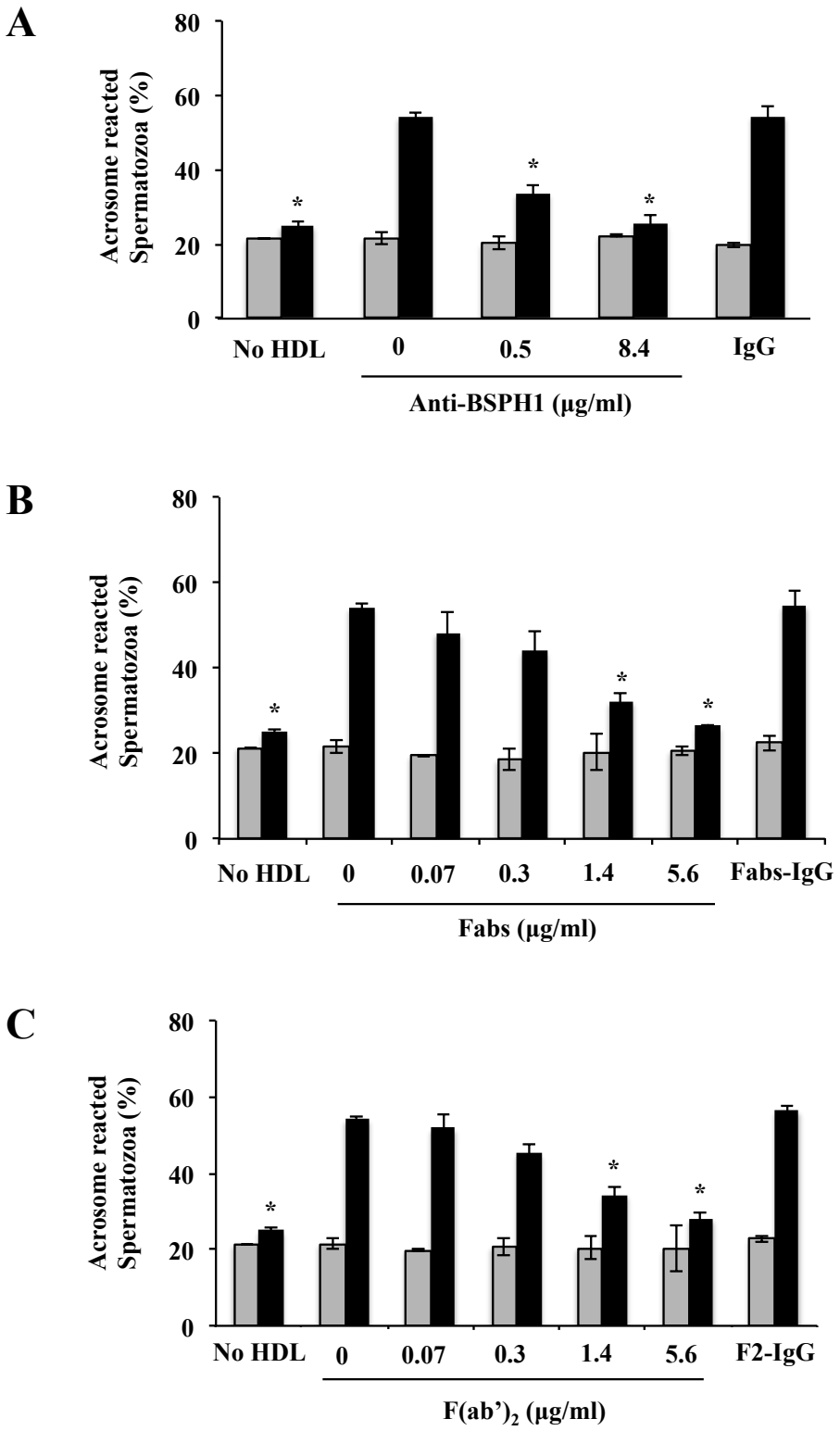

A
Acrosome reacted Spermatozoa (%)
80
60
40
20
0
No HDL
0
0.5
8.4
IgG
Anti-BSPH1 (µg/ml)
B
Acrosome reacted Spermatozoa (%)
80
60
40
20
0
No HDL
0
0.07
0.3
1.4
5.6
Fabs-IgG
Fabs (µg/ml)
C
Acrosome reacted Spermatozoa (%)
80
60
40
20
0
No HDL
0
0.07
0.3
1.4
5.6
F2-IgG
F(ab')2 (µg/ml)

FIGURE 4

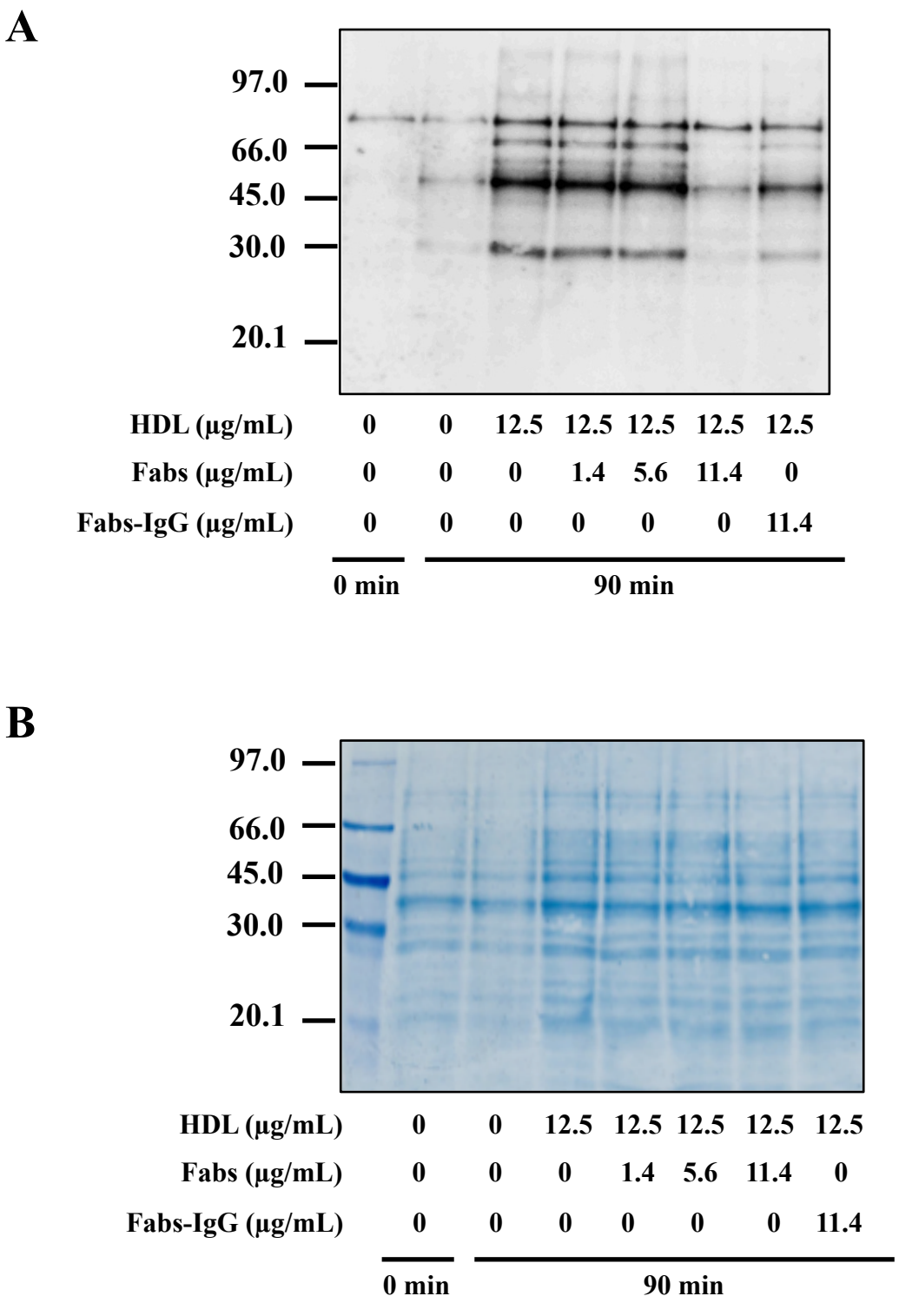

A
97.0
66.0
45.0
30.0
20.1
HDL (µg/mL) 0 0 12.5 12.5 12.5 12.5 12.5
Fabs (µg/mL) 0 0 0 1.4 5.6 11.4 0
Fabs-IgG (µg/mL) 0 0 0 0 0 0 11.4
0 min
90 min
B
97.0
66.0
45.0
30.0
20.1
HDL (µg/mL) 0 0 12.5 12.5 12.5 12.5 12.5
Fabs (µg/mL) 0 0 0 1.4 5.6 11.4 0
Fabs-IgG (µg/mL) 0 0 0 0 0 0 11.4
0 min
90 min

FIGURE 5

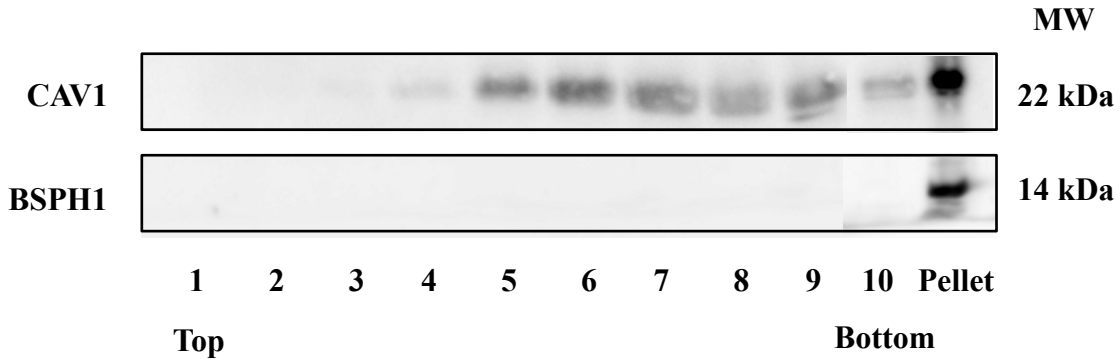


FIGURE 6

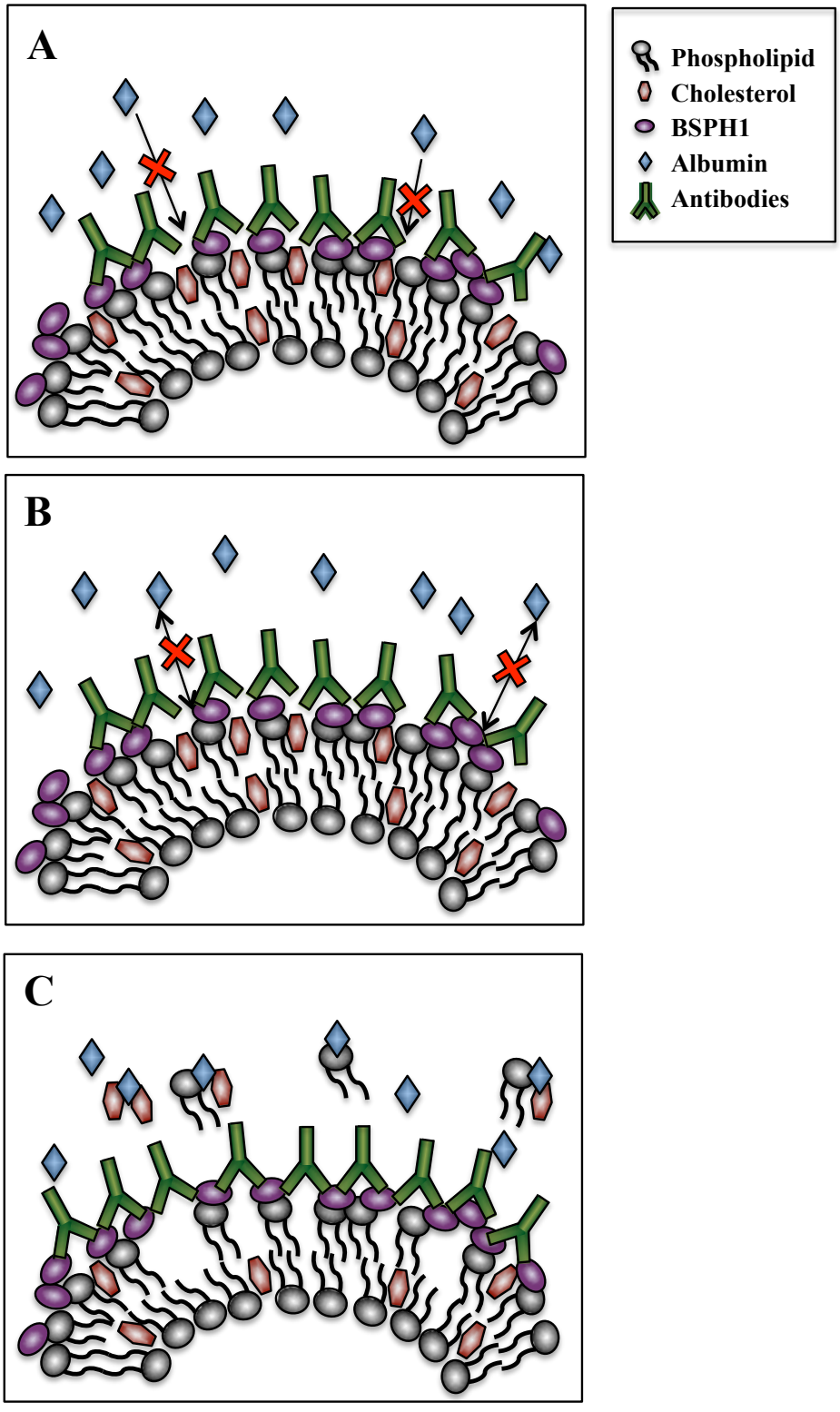
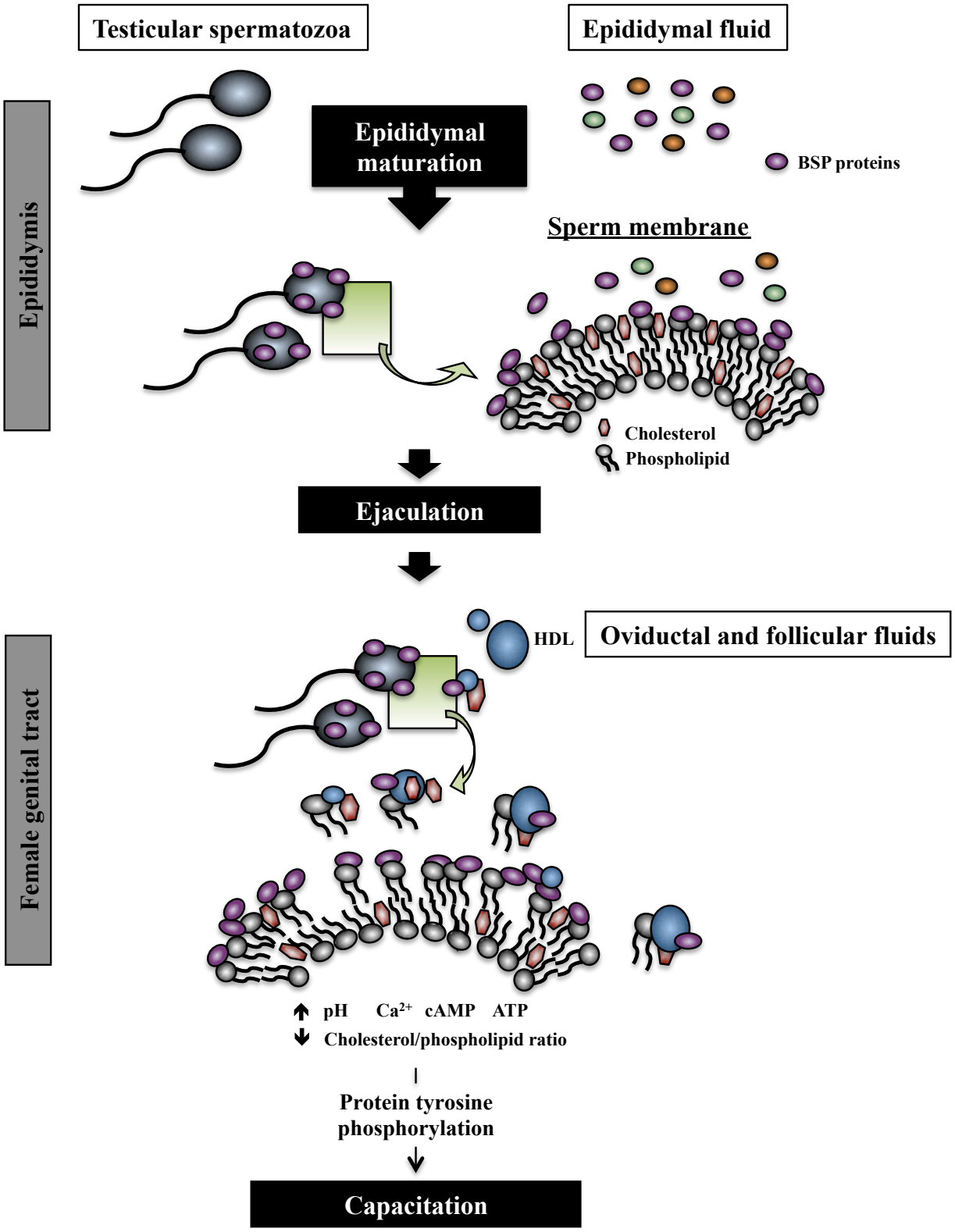


FIGURE 7


Testicular spermatozoa
Epididymal fluid
Epididymis
Epididymal maturation
BSP proteins
Sperm membrane
Cholesterol
Phospholipid
Ejaculation
HDL
Oviductal and follicular fluids
Female genital tract
pH Ca2+ cAMP ATP
Cholesterol/phospholipid ratio
Protein tyrosine phosphorylation
Capacitation

Discussion

Afin de pouvoir fusionner avec un ovocyte, les spermatozoïdes doivent subir une série d'étapes de maturation hautement régulée. Ces étapes incluent la maturation épididymaire, la capacitation et la réaction acrosomique. Plusieurs protéines du tractus génital mâle, dont les protéines de la famille des BSP, sont des joueurs clés pour la maturation des spermatozoïdes. En 2006, une analyse approfondie des génomes humain et murin a été réalisée afin d'identifier de nouveaux gènes codant pour des domaines Fn2 contenant les séquences consensus des protéines BSP. Ceci a permis l'identification de deux gènes homologues aux BSP chez la souris (*Bsph1* et *Bsph2*) et d'un gène chez l'humain (*BSPH1*) [161]. Cette étude a également démontré que ces nouveaux gènes sont plus fortement exprimés au niveau de l'épididyme, une première pour les protéines de la famille BSP. Durant la même période, plusieurs autres gènes et protéines BSP ont été identifiés dans les épididymes de mammifères dont les gènes *BSPH1* et *BSPH2* chez le taureau, le gène *BSP1* chez le chien et la protéine BSP1 chez le lapin [124, 164, 165].

Structurellement, toutes les BSP épididymaires identifiées ont un point en commun qui les distingue des autres protéines BSP: la présence d'une queue C-terminale. Chez les protéines BSP exprimées par les vésicules séminales, la partie C-terminale de la protéine se termine toujours par une cystéine alors que les protéines épididymaires contiennent entre 1 et 5 acides aminés suivant la dernière cystéine. Une autre différence a également été notée chez l'humain et la souris. Contrairement aux protéines bovines qui représentent plus de 50% des protéines totales du plasma séminal, seulement des traces des protéines homologues humaines et murines ont été retrouvées dans le plasma séminal de ces espèces.

En raison de ces différences, il était difficile de déterminer si les protéines BSP d'humain et de souris possèdent des rôles similaires et aussi importants dans la fécondation que celui jouer par les protéines BSP chez le taureau. Le but de cette thèse était de produire et purifier des protéines recombinantes BSP humaines et murines, d'étudier les caractéristiques de liaison de ces protéines et d'évaluer leurs rôles au niveau de certaines fonctions spermatiques.

1. Production et purification des protéines recombinantes

Les protéines BSP sont retrouvées chez l'humain et la souris en petite quantité. Pour cette raison, il n'était pas possible de les isoler du plasma séminal en quantité suffisante pour les caractériser et effectuer des expériences de fonctions. C'est pourquoi des protéines recombinantes ont été produites.

1.1 Système *E. coli* / pET32a

Les analyses de séquences de protéines BSP humaines et murines n'ont révélé la présence d'aucun site potentiel de glycosylation [161]. De plus, chez le bovin, la glycosylation ne semble pas essentielle pour la capacitation des spermatozoïdes, puisque BSP3, qui n'est pas glycosylé, est aussi actif que BSP1 et BSP5. Nous avons donc pu utiliser un système bactérien pour produire nos protéines recombinantes, même si les bactéries ne possèdent pas la machinerie nécessaire pour la glycosylation des protéines. Un système bactérien possède plusieurs avantages non négligeables pour la production de protéines recombinantes dont de faibles coûts, une croissance rapide des cellules et une facilité de manipulation. Une série d'expériences réalisées dans le laboratoire a permis de démontrer que l'utilisation d'un système combinant les bactéries *E. coli* origami B (DE3) pLysS et un vecteur d'expression pET32a donne les meilleurs résultats pour la production de protéines BSP recombinantes [166]. Ce système a donc également été utilisé pour le clonage de rec-BSPH1 et rec-BSPH2 de souris [166].

Les protéines BSP contiennent deux domaines Fn2 comprenant chacun deux ponts disulfures généralement difficiles à former correctement dans le cytosol plutôt réducteur des cellules *E. coli*. Pour surmonter ce problème, les cellules Origami B (DE3) pLysS sont souvent utilisées, puisqu'elles ont subi plusieurs modifications génétiques qui leur confèrent un cytosol plus oxydatif. [167]. Le vecteur d'expression pET32a est quant à lui fréquemment utilisé afin d'améliorer la solubilité des protéines recombinantes et d'éviter l'accumulation de celles-ci dans des corps d'inclusion. Ce vecteur permet l'ajout de trois étiquettes de fusion aux protéines recombinantes soit une étiquette thioredoxine, une étiquette $(His)_6$ et une étiquette S-tag. La thioredoxine est une protéine hautement soluble dans le cytoplasme des procaryotes, qui possède des propriétés de chaperon moléculaire et qui permet d'augmenter la solubilité des

protéines recombinantes auxquelles elle est attachée [168, 169]. Les deux autres étiquettes ajoutées servent plutôt à la purification des protéines recombinantes.

1.2 Purification et repliement

Les premières expériences utilisant le système origami / pET32a pour la production de rec-BSPH1 humaine ont mené à l'obtention de protéines pures et solubles [166]. Toutefois, des expériences subséquentes ont démontré que ces protéines formaient des agrégats de haut poids moléculaire et étaient incapable d'induire la capacitation des spermatozoïdes humains éjaculés (Article 2, Figure 1C). L'agrégation des protéines a été associée à un mauvais repliement de celles-ci. Des résultats similaires ont été obtenus lors d'expériences préliminaires avec rec-BSPH1 de souris. Nous avons donc dû développer une méthode permettant un meilleur repliement des protéines recombinantes, pour défaire les agrégats et obtenir des protéines fonctionnelles. La méthode qui a été développée se base sur des expériences réalisées par Lemercier et al [170]. Elle combine la purification des protéines par chromatographie d'affinité sur colonne d'ions métalliques immobilisés (IMAC) et le repliement des protéines sur colonne par un gradient d'urée décroissant.

Suite à l'expression des protéines dans les cellules origami, ces dernières sont lysées et de l'urée est ajoutée au lysat cellulaire afin d'obtenir une concentration finale de 6.0 M. Cet ajout permet de dénaturer les protéines et de défaire les agrégats tout en gardant les ponts disulfures intacts. Le lysat cellulaire est alors déposé sur la colonne d'IMAC pour séparer les protéines recombinantes des protéines bactériennes. Une fois les protéines liées à la colonne d'IMAC, un gradient d'urée décroissant de 6.0 M à 0 M est effectué sur une période d'environ 16 h. La liaison des protéines aux billes de la résine permet ainsi de séparer les protéines les unes des autres pour éviter la formation d'agrégats tandis que le gradient d'urée sur une longue période permet aux protéines d'adopter la conformation la plus stable.

Cette méthode a été utilisée pour la production des trois protéines recombinantes et, dans tous les cas, a permis de réduire la quantité d'agrégats de haut poids moléculaire présents initialement. Elle a également permis d'obtenir des protéines solubles, pures à environ 95% et adoptant différents niveaux d'oligomérisation (monomères, dimères, tétramères etc.) similaires à ceux observés chez les protéines BSP natives chez de taureaux, de porcs, d'étalons

et de boucs [117, 120, 150, 171, 172]. Ces protéines ont ensuite été utilisées pour tester les propriétés de liaison des protéines BSP humaines et murines.

2. Études de liaison

Les protéines BSP possèdent un éventail varié de ligands incluant la gélatine, les HDL, les LDL, les liposomes de PC et les GAG comme l'héparine et la CSB. Nous avons donc testé la liaison des protéines recombinantes humaines et murines à ces différents ligands pour savoir si elles partagent certaines caractéristiques avec les protéines des animaux de ferme.

2.1 Gélatine

Toutes les protéines BSP étudiées à ce jour ont la capacité de lier la gélatine ce qui est due à la présence des deux domaines de fibronectine. Afin de lier la gélatine, une conformation tridimensionnelle appropriée est requise [173]. Des études ont également montré que certains acides aminés hautement conservés sont essentiels pour la liaison de protéines contenant des domaines Fn2 à la gélatine [174]. La liaison des protéines recombinantes à la gélatine a été testée par chromatographie d'affinité. Dans les trois cas, entre 30 et 50% des protéines ont été retenues sur la colonne et éluées avec 6.0 M d'urée indiquant qu'une bonne partie des protéines possédait des domaines Fn2 correctement repliés.

Comme une analyse bioinformatique de l'hydrophobicité des protéines BSP a permis d'établir qu'elles ont besoin de se dimériser pour lier la gélatine (Article 3, Figure 4), il est possible qu'une partie des protéines n'ait pas lié la gélatine en raison d'une oligomérisation non appropriée. Il est aussi possible que, même suite au repliement sur colonne, une partie des protéines n'ait pas adopté une conformation adéquate. Toutefois, aucun lien direct n'a été établi entre la liaison à la gélatine et les fonctions biologiques des BSP. Donc, même si une partie des protéines recombinantes n'a pas montré d'affinité pour la gélatine, cela ne signifie pas que ces protéines sont inactives et n'induisent pas la capacitation. Des expériences réalisées avec une protéine recombinante BSP5 bovine, ont d'ailleurs montré que cette protéine liait faiblement la gélatine mais était quand même capable d'induire la capacitation aussi efficacement que la protéine native (Jois et *al.*, manuscrit en préparation).

2.2 GAG

Les GAG sont impliqués au niveau de plusieurs processus de la fécondation et la plupart des protéines BSP sont capable de s'y lier. Cette affinité est d'ailleurs exploitée pour isoler la protéine BSP1 du plasma séminal [129]. C'est pourquoi, la liaison des deux protéines recombinantes rec-BSPH1 et de rec-BSPH2 aux GAG, plus précisément la liaison à l'héparine et à la CSB, a également été testée par chromatographie d'affinité. Les trois protéines ne peuvent pas lier la CSB mais démontrent une forte affinité pour l'héparine.

La liaison aux GAG est attribuée à la présence d'acides aminés basiques dans les protéines BSP qui interagissent avec les charges négatives des GAG [136, 137]. Les protéines BSP épididymaires chez l'humain et la souris possèdent moins d'acides aminés basiques que les BSP des ongulés et ces acides aminés sont organisés différemment. Ces différences pourraient expliquer le manque d'interaction avec la CSB. Comme l'héparine est le GAG le plus négativement chargé, il est possible que les charges négatives additionnelles compensent pour la diminution d'acides aminés basiques, permettant la liaison des protéines recombinantes [175, 176].

Chez le taureau, les GAG sont impliqués dans la capacitation des spermatozoïdes ce qui n'est pas le cas chez l'humain ou chez la souris [177]. En effet, chez l'humain, certaines expériences tendent à démontrer que l'héparine peut induire directement la réaction de l'acrosome [178]. La liaison des BSP aux GAG chez ces deux espèces ne serait donc pas nécessaire pour l'initiation de la capacitation. Les GAG sont toutefois impliqués dans d'autres processus dont la liaison des spermatozoïdes à la ZP ce qui pourrait suggérer un rôle des BSP humaines et murines dans cette étape de la fécondation [50].

2.3 Liposomes de PC

Lors des premières études portant sur les protéines BSP bovines, notre équipe a démontré qu'elles peuvent interagir avec plusieurs types de phospholipides, ce qui leur permet de lier la membrane plasmique des spermatozoïdes. Ces études ont également montré que les BSP ont une affinité plus spécifique pour les phospholipides contenant des groupements choline [138]. Les liposomes de PC ont par la suite été largement utilisés pour étudier la liaison des BSP à ces phospholipides. En effectuant des expériences de co-sédimentation avec

des liposomes de PC, nous avons pu démontrer que les protéines rec-BSPH1 humaines et murines peuvent toutes deux lier les PC alors que la protéine rec-BSPH2 en est incapable. Même l'ajout de PE, de phosphatidylinositol ou de cholestérol aux liposomes n'a pas augmenté l'affinité de rec-BSPH2 pour les liposomes.

Une étude de cristallographie a permis de déterminer que la liaison entre la protéine BSP1 de taureau et les PC se fait via une interaction cationique entre le groupe amine quaternaire du groupement choline et un résidu tryptophane présent dans chacun des domaines Fn2 [141]. Cette étude a également montré que le lien est stabilisé et renforcé par les liens hydrogènes entre les groupements hydroxyles de trois résidus tyrosine dans chaque domaine de la protéine et les groupes phosphates des PC. En se basant sur les résultats de cette étude, des analyses bioinformatiques de liaison on été effectuées. Ces analyses ont révélé que chez la souris, la protéine rec-BSPH2 a une affinité pour le PC beaucoup plus faible que rec-BSPH1, surtout au niveau du deuxième domaine de fibronectine. Pour mieux comprendre cette différence d'affinité, les séquences des protéines humaines et murines ont été comparées avec les séquences des protéines bovines.

En alignant les séquences des protéines humaines et murines avec les séquences des protéines bovines, il est possible de voir que les BSPH1 de souris possède tous les résidus nécessaires pour la liaison au PC, alors que BSPH1 humaine et BSPH2 de souris ne possède pas deux et trois des six tyrosines essentielles pour la liaison respectivement (Figure 13). Pour BSPH1 humaine, une tyrosine par domaine Fn2 est manquante remplacée par une phénylalanine. Pour BSPH2, une tyrosine au niveau du premier domaine Fn2 est remplacée par une phénylalanine et deux tyrosines dans le second domaine Fn2 sont remplacées par une isoleucine et une glutamine respectivement. La tyrosine et la phénylalanine ont une structure très similaire qui ne diffère que par la présence d'un groupement hydroxyle. Le changement des résidus tyrosine des résidus phénylalanine pourrait donc être responsable d'une légère diminution de l'affinité pour les PC due à la perte d'un lien hydrogène sans causer de changements de structure majeurs de la protéine puisque rec-BSPH1 humain est capable de lier les liposomes de PC sans problèmes. Toutefois, au niveau des modifications du second domaine Fn2 de BSPH2, les changements d'acides aminés sont assez importants pour transformer légèrement la structure protéique et empêcher une bonne interaction avec les liposomes.

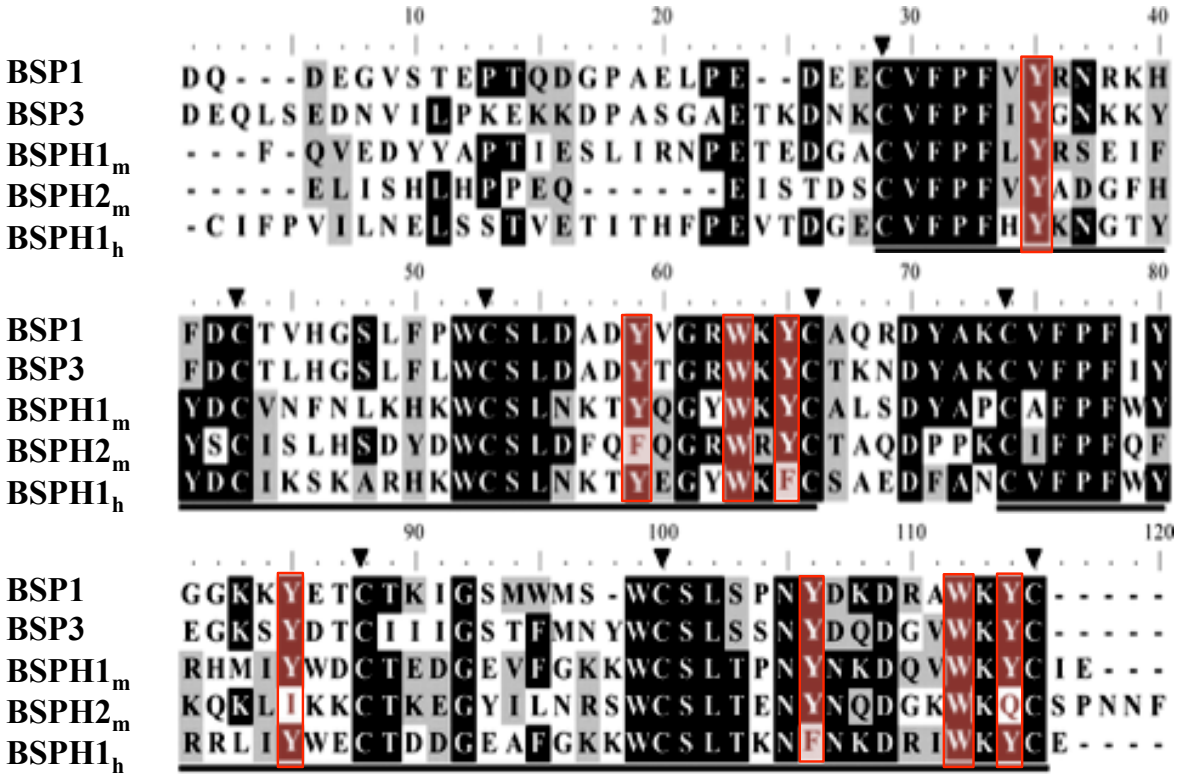


Figure 13: Alignement de séquence et identification des acides aminés essentiels pour la liaison aux PC. Les séquences des protéines humaine (h) et murines (m) ont été alignées avec les séquences de BSP1 et BSP3 bovines. Les résidus impliqués dans la liaison aux PC sont identifiés en rouge. Les domaines Fn2 sont soulignés [141, 161].

2.4 Spermatozoïdes

La fonction principale associée aux protéines BSP est la liaison à la membrane des spermatozoïdes afin de promouvoir la capacitation des spermatozoïdes. Les protéines BSP recombinantes humaines et murines ont été incubées avec des spermatozoïdes éjaculés humains ou des spermatozoïdes épididymaires murins selon la protéine. Dans les trois cas, les protéines ont été retrouvées dans le culot de spermatozoïdes après centrifugation. Comme les spermatozoïdes épididymaires et les spermatozoïdes éjaculés ont déjà été en contact avec les protéines BSP natives exprimées dans les épididymes des deux espèces, les protéines natives ont également été détectées dans les culots de spermatozoïdes.

Les deux protéines recombinantes BSPH1 ont la capacité de lier les liposomes de PC. Leur liaison aux spermatozoïdes n'était donc pas surprenante. Toutefois la liaison de rec-BSPH2 aux spermatozoïdes était une légère surprise. Comme les protéines BSP ont la capacité d'interagir ensemble et de se lier les unes aux autres, nous avons pensé que lors de l'incubation avec les spermatozoïdes, les protéines rec-BSPH2 pouvaient se lier aux protéines natives BSPH1 se trouvant déjà à la surface des spermatozoïdes. Toutefois, cette théorie a été démentie, puisque l'incubation de spermatozoïdes avec des anticorps spécifique pour BSPH1, afin de bloquer l'accès aux protéines BSPH1 natives, avant l'incubation des spermatozoïdes avec rec-BSPH2 n'empêche pas la liaison de rec-BSPH2 aux spermatozoïdes (résultats non publiés).

Il existe donc deux hypothèses encore valides pour expliquer la liaison de rec-BSPH2 aux spermatozoïdes murins. Il est premièrement possible l'organisation hétérogène des phospholipides dans la membrane plasmique, qui est différente de l'organisation des lipides dans les liposomes expérimentaux, contienne différents micro-domaines favorisant la liaison de rec-BSPH2 aux cellules. La deuxième hypothèse possible est que rec-BSPH2 pourrait avoir d'autres partenaires protéiques d'interaction à la surface des spermatozoïdes. Toutefois, jusqu'à ce jour, aucune protéine de spermatozoïdes interagissant avec des membres de la famille BSP n'a été identifiée.

3. Immunolocalisation

La membrane des spermatozoïdes est divisée en plusieurs régions afin de permettre les différents processus de la fécondation. Chaque région membranaire, et les protéines qui s'y trouvent, est impliquée au niveau de différents processus. Sur la tête du spermatozoïde, les protéines recouvrant la région acrosomique sont généralement impliquées dans la capacitation et l'initiation de la réaction de l'acrosome. Les protéines au niveau du segment équatorial sont impliquées au niveau de l'interaction et de la fusion du spermatozoïde et de l'ovocyte alors que les protéines de la région post-acrosomique servent à la capacitation et la l'interaction avec l'ovocyte. Au niveau de la queue du spermatozoïde, les protéines servent majoritairement à l'apport d'énergie et au développement de la motilité.

Une fois la liaison des protéines BSP humaines et murines aux spermatozoïdes confirmée, il devenait intéressant de voir par immunodétection sur quelles régions de la

membrane plasmique ces protéines se lient. Les résultats ont montré des patrons légèrement différents pour l'humain et la souris (Figure 14). Chez l'humain, les protéines BSPH1 natives et recombinantes ont, toutes deux, été observées majoritairement au niveau de la tête du spermatozoïde et plus spécifiquement sur le segment équatorial, la région post-acrosomique et le cou du spermatozoïde suggérant des rôles au niveau de la capacitation et possiblement de l'interaction spermatozoïde-ovocyte. Chez la souris, les protéines BSPH1 et BSPH2 natives et recombinantes se retrouvaient autant au niveau de la queue du spermatozoïde qu'au niveau de la tête, liant la pièce médiane du spermatozoïde et les trois régions de la tête.

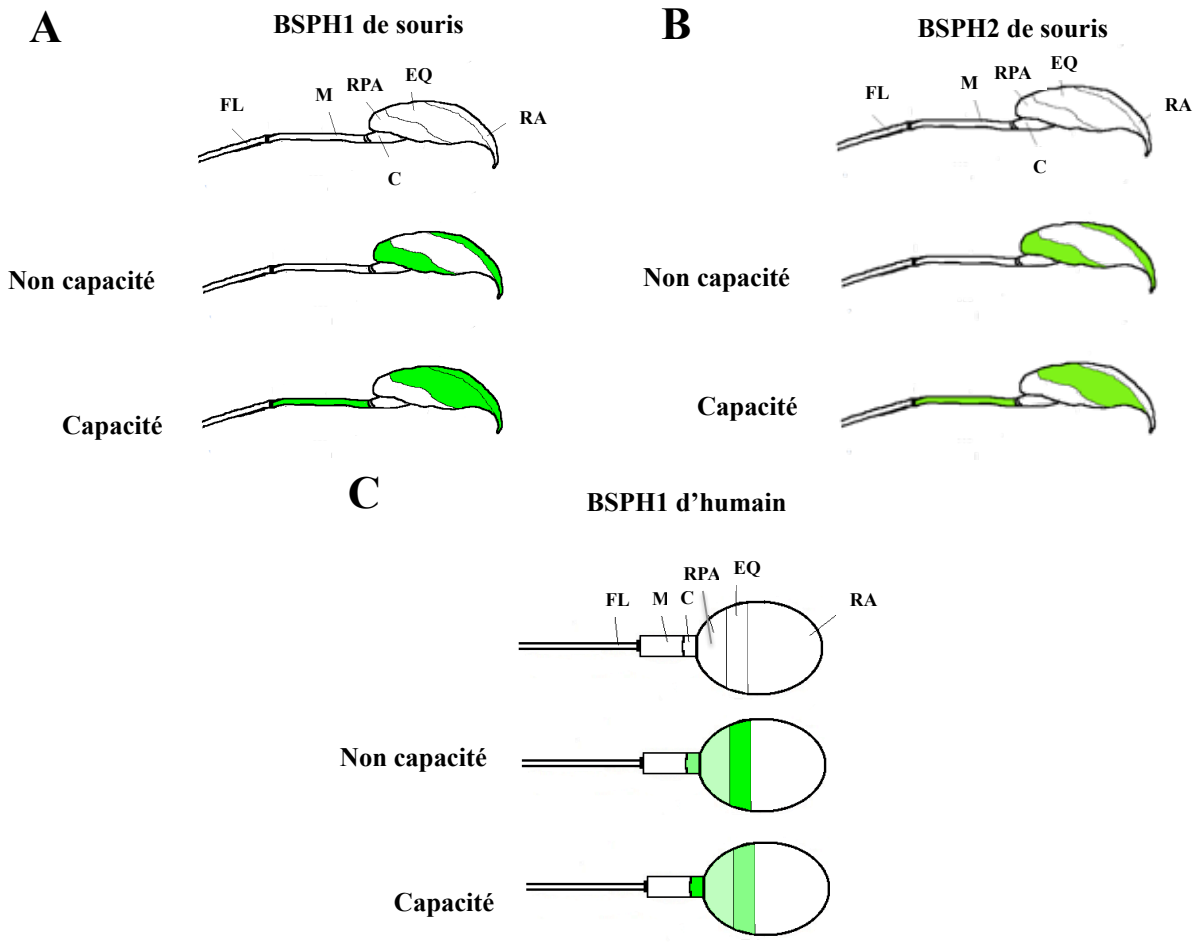


Figure 14: Localisation des protéines BSP à la surface des spermatozoïdes humains et murins. (A) BSPH1 de souris, (B) BSPH2 de souris et (C) BSPH1 d'humain. (C, Cou ; EQ, segment équatorial ; FL, flagelle ; M, pièce médiane ; RA, région acrosomique ; RPA, région post-acrosomique).

Chez les deux espèces, un changement de localisation des protéines à la surface des spermatozoïdes a été observé suivant la capacitation. Chez la souris, au niveau de la tête du spermatozoïde, les protéines se déplacent des régions acrosomique et post-acrosomique au segment équatorial suivant la capacitation. Une partie des protéines BSPH1 demeure sur la région acrosomique suivant la capacitation, alors que la protéine BSPH2 disparaît complètement de cette région. Ce réarrangement renforce l'hypothèse selon laquelle les protéines BSP pourraient jouer un rôle lors de l'interaction entre le spermatozoïde et l'ovocyte, puisque la région équatoriale de la tête est le lieu de contact avec l'ovocyte.

Au niveau de la queue des spermatozoïdes de souris, un signal plus fort sur la pièce médiane a été observé suite à une incubation en présence d'albumine. Un patron similaire a été observé avec les spermatozoïdes humains puisque, suite à quatre heures d'incubation en conditions capacitantes, un signal plus fort était observé sur le cou du spermatozoïde. Cela suggèrerait un rôle des BSP au niveau de la motilité des spermatozoïdes et plus spécifiquement au niveau de l'hyperactivation puisque le réarrangement apparait suivant une incubation en milieu capacitant.

4. Fonctions spermatiques

Les protéines BSP retrouvées chez les ongulés jouent une panoplie de rôles au court de la fécondation, étant impliquées au niveau de la formation du réservoir de spermatozoïdes, de la motilité des spermatozoïdes, de la capacitation et possiblement au niveau de régulation du volume cellulaire et de l'interaction spermatozoïde-ovocyte. L'effet des protéines recombinantes rec-BSPH1 et rec-BSPH2 sur la capacitation, la motilité et la réaction acrosomique des spermatozoïdes humains et murins a donc été testé.

4.1 Capacitation

4.1.1 Méthodologie

Les changements qui se produisent chez les spermatozoïdes lors de la capacitation ne sont pas faciles à détecter par microscopie. Il est possible de différencier les spermatozoïdes capacités des spermatozoïdes non capacités en utilisant la chlortétracycline (CTC), une sonde fluorescente qui adopte des patrons de coloration différents selon l'état du spermatozoïde.

Cette méthode est plus souvent utilisée pour les spermatozoïdes bovins et porcins, mais peut également être utilisée chez l'humain et la souris [179, 180]. Cette méthode comporte certains désavantages. Par exemple, chez la souris, la différence de patron entre les spermatozoïdes capacités et non capacités est souvent difficile à voir. La courte durée de fluorescence de la CTC ajoute également un défi supplémentaire lors de l'utilisation de cette méthode. C'est pour quoi, lors de nos études, nous avons utilisé la CTC, la seule méthode permettant de mesurer la capacitation directement, uniquement pour confirmer le bon fonctionnement des autres méthodes utilisées.

Chez l'humain et la souris, les deux méthodes les plus souvent utilisées pour déterminer l'état capacité des spermatozoïdes sont l'induction de la réaction acrosomique et la détection par Western blot de l'augmentation de la phosphorylation des résidus tyrosine. La première méthode a été utilisée pour la majorité des expériences présentées dans cette thèse. Seuls les spermatozoïdes capacités peuvent subir la réaction acrosomique. Donc, afin de déterminer l'état capacité des spermatozoïdes, il est possible d'incuber ceux-ci en présence d'ionophore de calcium pour induire la réaction acrosomique et de vérifier par la suite si l'acrosome est intact. Le statut de l'acrosome est vérifié chez la souris en utilisant une coloration au bleu de Coomassie alors que chez l'humain il est généralement vérifié grâce à l'agglutinine de *Pisum sativum* couplée au fluorochrome FITC (PSA-FITC).

4.1.2 Protéines recombinantes et capacitation

Une fois les propriétés de liaison des protéines recombinantes établies, l'effet des protéines rec-BSPH1 et rec-BSPH2 sur la capacitation des spermatozoïdes a été évalué. Les résultats obtenus montrent que l'addition de rec-BSPH1 humain à des spermatozoïdes humains éjaculés et l'addition de rec-BSPH1 murin à des spermatozoïdes épididymaires de souris cause une augmentation du nombre de spermatozoïdes qui subissent la réaction acrosomique suite à l'ajout d'ionophore de calcium. Aucune augmentation n'a été observée en absence d'ionophore ce qui démontre une augmentation de la capacitation en présence des protéines rec-BSPH1. Cet effet sur la capacitation était dose-dépendant. Ces résultats sont très similaires à ceux obtenus chez les animaux de ferme. Nos expériences ont également pu démontrer que les protéines BSP ne sont pas spécifiques à une espèce et peuvent la capacitation de spermatozoïdes d'autres espèces. En effet, l'ajout de protéine BSP1 bovine à

des spermatozoïdes humains et murins ou encore l'ajout de protéines rec-BSPH1 de souris à des spermatozoïdes humain permet d'induire la capacitation de ces spermatozoïdes (résultats non publiés).

Comme lors des expériences de liaison aux liposomes de PC, des résultats différents ont été obtenus pour la protéine rec-BSPH2 lors des expériences de capacitation. En effet, cette protéine n'a causé aucune augmentation du pourcentage de spermatozoïdes murins ayant subit la réaction acrosomique en présence ou en absence d'ionophore. Ce manque d'activité de la protéine BSPH2 chez la souris peut être dû à la faible affinité de liaison de son domaine Fn2-B pour les phospholipides, puisque plusieurs études ont démontré que chez les BSP les deux domaines sont nécessaires pour l'induction de la capacitation [181].

L'effet des protéines rec-BSPH1 sur l'augmentation de la phosphorylation des résidus tyrosines des spermatozoïdes humains et murins a également été évalué. Dans les deux cas, aucun changement significatif n'a été observé entre les spermatozoïdes incubés seuls et les spermatozoïdes incubés en présence de protéines recombinantes (résultats non publiés pour la souris). Ces résultats semblent démontrer que l'ajout de protéines recombinantes seules permet l'induction de la capacitation sans effet au niveau de la signalisation cellulaire. Ces résultats ressemblent à ceux obtenus chez le bovin lors de la capacitation induite par les BSP en présence de HDL. Dans ces conditions, les BSP et les HDL causent un efflux de cholestérol et de phospholipides, mais ne causent aucune augmentation de la phosphorylation [148]. Ces résultats suggèrent donc une implication des protéines BSPH1 de souris et d'humains au niveau de l'efflux de cholestérol et de phospholipides lors de la capacitation.

4.1.3 BSPH1 murine et HDL

Ces expériences, bien qu'informatives au niveau du rôle des BSP dans la capacitation, ont été réalisées en présence de concentrations élevées de protéines recombinantes, loin des concentrations retrouvées dans les conditions biologiques. Nous avons donc poussé l'étude plus loin afin de tester le rôle de la protéine BSPH1 native de souris dans la capacitation. Pour ce faire, des anticorps complets et des fragments d'anticorps spécifiques contre la protéine BSPH1 ont été utilisés.

Chez le bovin, les protéines BSP induisent la capacitation via des interactions avec les HDL et/ou avec l'héparine alors que chez la souris, seul les HDL sont impliquées dans la

capacitation, l'héparine étant impliquée dans la réaction acrosomique et l'interaction spermatozoïde-ovocyte [182]. Nous avons donc testé s'il était possible d'inhiber la capacitation induite par les HDL en bloquant l'accès aux protéines BSPH1 à la surface des spermatozoïdes à l'aide d'anticorps. Les résultats obtenus démontrent que l'ajout d'anticorps, ou de fragments d'anticorps spécifiques pour BSPH1 lors de l'incubation de spermatozoïdes en présence de HDL, permet de diminuer la quantité de spermatozoïdes ayant subi la réaction acrosomique suivant l'incubation en présence d'ionophore de calcium. La présence de fragments d'anticorps anti-BSPH1 lors de l'incubation des spermatozoïdes avec les HDL a également causé une diminution significative des niveaux de phosphorylation des tyrosines associés à la présence de HDL. Ces résultats suggèrent qu'une interaction directe entre les HDL et la protéine BSPH1 pourrait être nécessaire pour la capacitation des spermatozoïdes chez la souris.

4.1.4 Modèle proposé du rôle de BSPH1 dans la capacitation

L'effet des anticorps complets anti-BSPH1 sur la capacitation induite par l'albumine a également été testé. Ces expériences ont démontré que BSPH1 n'intervient pas directement dans les processus de capacitation induite par l'albumine. Toutefois, les anticorps anti-BSPH1 complets sont capables de stabiliser suffisamment la membrane des spermatozoïdes de manière à inhiber complètement l'effet de l'albumine. Ces résultats suggèrent un rôle d'agents décapacitants pour les protéines BSP. Ce rôle pourrait également expliquer la liaison de BSPH1 sur la région post-acrosomique de la tête des spermatozoïdes non capacités, puisque cette section du spermatozoïde est la partie la plus faible en cholestérol et donc la plus fluide de la cellule [43].

Tous les résultats décrits précédemment ont mené au développement d'un modèle expliquant la fonction de BSPH1 murine dans la capacitation des spermatozoïdes (article 4, figure 7). Lors de la maturation épididymaire, les spermatozoïdes entre en contact avec BSPH1 qui se lie à leur surface via une interaction avec les phospholipides portant des groupements choline. Cette interaction stabilise la membrane et prévient une capacitation et/ou une réaction acrosomique prématurée. Suivant l'éjaculation, les spermatozoïdes se retrouvent dans le tractus génital femelle, puis dans l'oviducte où ils entrent en contact avec les HDL. Les protéines BSPH1 à la surface des spermatozoïdes interagissent avec les HDL pour leur

transférer des phopholipides et du cholestérol causant une diminution du ratio cholestérol/phospholipides, ce qui déstabilise la membrane. Cela entraîne alors une augmentation du pH, du calcium et de l'AMPc intracellulaire qui augmente la phosphorylation des tyrosines et mène à l'état capacité des spermatozoïdes.

4.2 Autres fonctions

Pour l'instant, aucune autre fonction n'a été attribuée aux protéines BSP chez l'humain et la souris. Aucun effet n'a été observé sur les paramètres de motilité testés chez l'humain, mais il est possible que l'action des BSP sur cette fonction spermatique nécessite la présence d'albumine ou de HDL.

Le rôle de BSP au niveau des différentes étapes de l'interaction spermatozoïde-ovocyte n'a pas encore été évalué mais les expériences de localisation suggèrent fortement une implication des protéines murines dans ces étapes. Toutefois, les protéines impliquées dans l'interaction avec l'ovocyte se trouvent généralement au niveau des radeaux lipidiques de la membrane ce qui n'est pas le cas de BSPH1 de souris [183].

5. Sous-familles des BSP

L'analyse phylogénétique des protéines de la famille des BSP a permis de classer les différents membres en trois sous-familles soit BSP, BSPH1 et BSPH2 (Figure 15). Les membres de la famille sécrétés au niveau des vésicules séminales font tous partie de la sous-famille BSP1 alors que les protéines épididymaires sont réparties dans les trois sous-familles. Lors de nos études, les résultats ont clairement démontré que les protéines BSPH1 humaine et murine, qui sont orthologues et font partie de la même sous-famille, sont très similaires partageant les mêmes caractéristiques de liaisons et les mêmes caractéristiques fonctionnelles. La protéine BSPH2 quant à elle agit plus comme le mouton noir de la famille des BSP puisqu'elle affiche des caractéristiques uniques, tel son incapacité à lier les liposomes de PC et son incapacité à induire la capacitation. Les caractéristiques de ces trois protéines sont également différentes de celles de la protéine épididymaire BSP1 de lapin qui est une grosse protéine comparativement aux autres BSP (52 kDa) et qui se lie uniquement au flagelle du spermatozoïde. Les résultats obtenus au cours de cette thèse révèlent donc que les propriétés et

fonctions des BSP semblent être déterminées plus par la sous-famille dont elles font partie que par l'organe dans lequel elles sont exprimées.

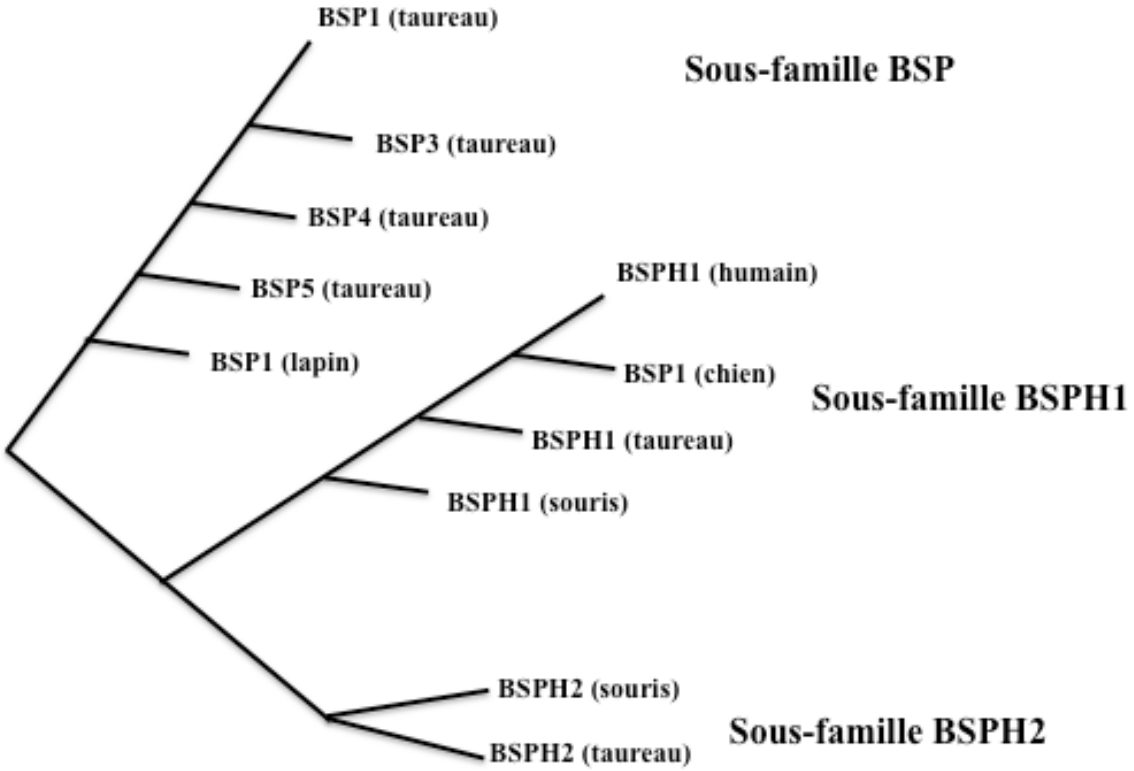


Figure 15: Analyse phylogénétique des protéines BSP. (Adapté de [115, 124])

6. BSP et fertilité masculine

En général, la fertilité masculine est testée en clinique via un spermogramme afin de vérifier le compte spermatique, la morphologie des cellules et la motilité des spermatozoïdes. Des tests sanguins, des échographies testiculaires et des tests de fragmentation de l'ADN des spermatozoïdes peuvent également être effectués [9]. Toutefois, la capacité des spermatozoïdes à subir la capacitation et la réaction acrosomique est rarement évaluée. De ce fait, un homme ayant un problème de fertilité relié à ces étapes de la fécondation reçoit souvent un diagnostic d'infertilité idiopathique. Comme 30 à 40% des cas d'infertilité masculine sont de causes inconnues, une meilleure compréhension des mécanismes à la base de la capacitation et de la réaction acrosomique pourrait permettre d'identifier de nouvelles causes d'infertilité.

Les résultats obtenus et présentés dans cette thèse de doctorat suggèrent fortement un rôle pour les protéines BSPH1 humaines et murines au niveau de la capacitation des spermatozoïdes et suggèrent même que ces protéines pourraient être essentielles pour la fertilité. La génération de souris KO pour les gènes *Bsph1* et *Bsph2* est présentement en cours dans le laboratoire ce qui nous permettra bientôt de connaître l'importance réelle des protéines BSP au niveau de la fertilité murine. Comme les protéines BSPH1 humaines et murines sont orthologues, les résultats des études de souris KO devraient également refléter l'importance de BSPH1 humaine dans la fertilité masculine. Toutefois, plusieurs exemples de gènes importants pour la fertilité murine sans impact connu sur la fertilité humaine ont été décrits au cours des dernières années. Tel est le cas de la protéine IZUMO. En effet, les souris KO pour le gène *IZUMO* sont complètement infertiles dû à un problème de fusion entre le spermatozoïde et l'ovocyte [184]. À ce jour, aucun cas d'infertilité masculine n'a été associé à une protéine IZUMO non fonctionnelle [185]. C'est pourquoi des études sont également en cours afin de déterminer si des mutations sont présentes dans le gène humain *BSPH1* chez des patients souffrant d'infertilité idiopathique et si ces mutations sont la cause de l'infertilité observée. Les résultats de ces deux études devraient nous permettre d'établir clairement si l'absence de protéines BSP fonctionnelle peut être une cause d'infertilité masculine. Le cas échéant, des tests diagnostiques et des traitements adaptés pourront être développés. Si les BSP sont essentielles à la fertilité, elles pourraient également être utiles pour le développement de contraceptifs masculins.

6.1 Tests diagnostiques et développement de traitements

Si une corrélation est établie entre la présence de mutations dans le gène *BSPH1* humain et l'infertilité masculine, des tests diagnostiques et des traitements pourraient être développés. Les tests diagnostiques pourraient entre autres inclurent des tests de PCR à la recherche de mutations connues dans le gène *BSPH1* ou encore le développement d'essais ELISA (enzyme-linked immunosorbant assay) afin de quantifier la protéine BSPH1 dans les éjaculats de patients infertiles. Puisque les protéines BSP semblent être importantes au niveau de la capacitation, une étape post-éjaculatoire, l'ajout de protéines fonctionnelles aux spermatozoïdes éjaculés de patients infertiles pourrait permettre l'induction de la capacitation des spermatozoïdes et le retour d'une fertilité normale pour ces patients. Les spermatozoïdes

pourraient alors être utilisés pour la FIV ou encore injectés directement par IA, ce qui réduirait considérablement les coûts associés aux techniques de reproduction assistée.

6.2 Développement de contraceptifs

La protéine BSPH1 pourrait également devenir une cible de choix pour le développement de contraceptifs masculins. Plusieurs facteurs doivent toutefois être pris en compte afin de développer des nouveaux contraceptifs. Ces derniers doivent préférablement être efficaces, spécifiques et réversibles. Dans le cas du développement d'un contraceptif ayant pour cible BSPH1, une méthode possible serait l'utilisation d'une petite molécule ciblant les domaines Fn2 de la protéine, puisque ceux-ci sont responsables de l'interaction des BSP avec les phospholipides composant la membrane des spermatozoïdes. La protéine BSPH2 de souris ne peut lier les liposomes de PC ou induire la capacitation des spermatozoïdes. Par conséquent, une étude plus approfondie de cette protéine pourrait aider l'identification des régions cibles pour l'inhibition de la fonction de BSPH1 humaine. Plusieurs protéines humaines contiennent également des domaines de fibronectine. Quelques précautions devraient donc être prises afin de s'assurer la spécificité du contraceptif ciblant les protéines BSP.

Un autre détail important à prendre en compte lors du développement de contraceptif ciblant des facteurs épididymaires est la présence d'une barrière physique et physiologique entre le sang et l'épididyme. Cette barrière doit en effet être traversée par les molécules contraceptives pour qu'elles soient efficaces. Cette barrière peut être un inconvénient au développement d'un contraceptif mais peut également être un avantage. Plusieurs canaux et transporteurs ont été étudiés au niveau de l'épididyme et pourraient être exploités afin de faire entrer les contraceptifs dans l'organe. En ciblant des transporteurs spécifiques à l'épididyme, une certaine spécificité serait ainsi conférée au contraceptif en question [186].

Une autre approche pouvant être considérée pour le développement de contraceptifs est l'immunocontraception. On sait depuis longtemps que l'injection de spermatozoïdes humains chez l'homme ou la femme peut induire une réponse immunitaire et réduire la fertilité de l'individu en question [187, 188]. Plusieurs études visant à développer des vaccins ciblant soit des hormones impliquées au niveau de la production de gamètes ou encore des protéines impliquées dans les fonctions des gamètes (antigènes de spermatozoïdes, protéines de la ZP)

ont été réalisées [189]. Les résultats de ces études tendent à démontrer que les protéines de spermatozoïdes sont des meilleures cibles que les hormones sexuelles qui sont impliquées au niveau de plusieurs fonctions physiologiques [189]. Les recherches actuelles visent donc à identifier des antigènes spécifiques aux spermatozoïdes et aux systèmes reproducteurs pour assurer une grande spécificité d'action de l'immunisation. Plusieurs protéines testiculaires (ex : IZUMO) et épididymaires (ex : P34H et CRISP) ont déjà été proposées comme candidats potentiels pour le développement de vaccins [190-192]. Comme l'utilisation d'anticorps contre BSPH1 de souris permet d'inhiber la capacitation *in vitro*, l'immunocontraception pourrait être une approche à considérer pour le développement d'un contraceptif ciblant BSPH1.

Conclusion et perspectives

Au cours de cette thèse, plusieurs avancées dans l'étude des protéines homologues aux BSP exprimées dans les épididymes humains et murins ont été réalisées. Premièrement une méthode de production de protéines BSP recombinantes fonctionnelles a été développée ce qui constitue en soit un grand pas permettant une étude plus approfondie des protéines Binder of SPerm. Deuxièmement, nous avons démontré que les protéines BSP épididymaires partagent plusieurs caractéristiques avec leurs comparses exprimés dans les vésicules séminales. Ces trois protéines recombinantes (BSPH1 humaine, BSPH1 murine et BSPH2 murine) peuvent lier la gélatine, l'héparine et la membrane des spermatozoïdes. Les protéines BSPH1 sont également capables de lier les liposomes de PC et d'induire la capacitation. Nous avons également identifié pour la première fois un membre de la famille BSP incapable de lier les liposomes de PC ou d'induire la capacitation des spermatozoïdes. Cette découverte ouvre plusieurs portes vers l'étude plus poussée des séquences des protéines BSP afin d'identifier les d'acides aminés essentiels pour l'exécution de leurs fonctions. Finalement, nos études ont permis de montrer que la protéine BSPH1 de souris semble essentielle pour la capacitation induite par le HDL. Comme cette protéine est orthologue à la protéine BSPH1 humaine qui est le seul membre de la famille BSP chez l'humain, cela ouvre également la porte à des études visant à déterminer si des mutations au niveau du gène *BSPH1* pourrait être à l'origine de problèmes de fertilité chez les hommes.

Les études décrites dans cette thèse ont fait grandement avancer nos connaissances sur les membres épididymaires de la famille des BSP, mais beaucoup reste encore à découvrir. Dans le futur, des expériences utilisant les protéines recombinantes et les anticorps anti-BSP devraient aider à faire la lumière sur le rôle des protéines BSP au niveau de l'interaction spermatozoïde-ovocyte et au niveau de la motilité des spermatozoïdes en présence d'agents capacitants.

La génération de souris KO et l'étude de mutations chez l'humain permettront enfin de déterminer si les protéines BSP sont essentielles à la fertilité humaine et murine. Les souris KO devraient également permettre de répondre à plusieurs questions sur l'implication des BSP au niveau des modifications membranaires qui se déroulent durant la maturation épididymaire,

au niveau de la capacitation des spermatozoïdes et au niveau de l'interaction spermatozoïde-ovocyte.

Toutes ces études pourraient mener à l'identification de nouveaux facteurs importants pour la fertilité humaine, le développement de test diagnostiques et de nouveaux traitements et pourrait même mener au développement de contraceptifs masculins ayant pour cible les protéines BSP.

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