APPENDIX C

BOVINE SEMINAL PLASMA PROTEINS BSP A3 AND BSP 30KDA SHARE FUNCTIONAL ROLES WITH PDC-109 IN PROVIDING PROTECTIVE EFFECTS TO SPERM

Abstract

Upon ejaculation, bull sperm are exposed to the secretions of the seminal vesicles, prostate, and accessory glands and become coated with various proteins, including a family of proteins called BSPs (bovine seminal plasma proteins), comprising BSP A1/A2, BSP A3 and BSP 30kDa. Previously, we demonstrated that BSP A1/A2 (PDC-109) enables epididymal sperm to bind to explants of oviductal epithelium, and competitively inhibits binding of ejaculated sperm. Because the BSPs share significant sequence homology, we investigated if BSP A3 and BSP 30 kDa were also effective at mediating binding of sperm to oviductal epithelium. Both BSP A3 and BSP 30kDa significantly increased binding density of epididymal sperm. This effect was not observed when a protein of similar size and charge (myosin light chain) was added to sperm. Likewise, BSP A3 and BSP 30kDa were as effective as PDC-109 in competitively reducing binding density of ejaculated sperm. Because the fertile life of sperm has been shown to be extended by binding to oviductal epithelium, we sought to determine if maintenance of sperm motility was attributed to the action of the BSPs. The % progressive motility of fresh epididymal sperm treated with PDC-109, BSP A3 or BSP 30kDa was significantly greater than that of untreated sperm following eight hours co-incubation with vesicles of apical plasma membrane derived from bovine isthmic oviductal epithelium. Linear regression analyses indicated that

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1 This manuscript is a collaboration with TanYa M. Gwathmey, George G. Ignatz, Puttaswamy Manjunath, and Susan S. Suarez at the Cornell Veterinary School. This appendix represents a draft form of the manuscript. My contribution to this manuscript includes generating and verifying the comparative structural models for BSPA3 and BSP 30kDa and making predictions based upon these models, of their functional similarities and differences in sperm and oviductal membrane interactions.
each BSP produced a dose-dependent effect on maintenance of progressive motility. These results indicate that BSP A3 and BSP 30kDa can also mediate sperm binding to oviductal epithelium and that each of the BSPs play a role in maintenance of sperm motility. Taken together, these data suggest that, in vivo, binding of sperm to oviductal epithelium and subsequent preservation of sperm during storage is mediated by seminal plasma proteins.

**Introduction**

In order for fertilization to occur, sperm must be available and in the proper physiological state, i.e., still motile and not acrosome reacted when ovulation occurs. To ensure that sperm are available to fertilize the egg following ovulation, an oviductal sperm reservoir has evolved and has been studied in several species of animals (reviewed in Suarez, 1998; reviewed in Suarez, 2002). However, to date, most information is known about the sperm reservoir found in cattle. The reservoir serves to maintain the fertility of sperm until ovulation by regulating capacitation (Dobrinski et al., 1996b), maintain sperm motility (Adams et al., 2000; Chian and Sirard, 1995) as well as to prevent polyspermy by allowing only a small number of sperm to reach the oocyte as it enters the ampulla (Hunter and Leglise, 1971; Polge et al., 1970).

How is the reservoir formed? Once the sperm are deposited in the female reproductive tract, they become trapped in the initial segment of the oviduct by binding to epithelial cells (Lefebvre et al., 1995). Binding occurs as a result of carbohydrate-based recognition. That is, a lectin-like protein present in the outer leaflet of the sperm plasma membrane recognizes and binds to a carbohydrate-containing moiety expressed on the surface of the oviductal epithelium. Several species have been identified which exhibit carbohydrate-mediated binding of sperm to
the oviductal epithelium (hamsters (DeMott et al., 1995), horses (Ball et al., 1997; Dobrinski et al., 1996a), cattle (Lefebvre et al., 1997; Suarez, 1998), pigs (Jansen et al., 2001; Partridge et al., 1987) and rats (Cortes et al., 2004). For each species evaluated, there exists a carbohydrate-specificity of binding. For example, binding of hamster sperm to homologous epithelium is mediated by sialic acid (DeMott et al., 1995), whereas galactose mediates binding of equine sperm (Lefebvre and Suarez, 1996). In boar sperm, however, binding to epithelium is competitively inhibited by maltose, lactose and mannose (Partridge et al., 1987). In cattle, terminally expressed fucosylated molecules are the ligands to which bovine sperm attach (Lefebvre et al., 1997; Suarez, 1998). Previously, we isolated a protein on bovine sperm which recognizes and binds to fucosylated oviductal ligands. This protein was identified as PDC-109 (Protein with N-terminus aspartic acid, D, and carboxy terminus Cystein, having 109 amino acids) as determined by amino acid sequencing (Ignotz et al., 2001).

PDC-109 is one of three major heparin-binding proteins contained within bovine seminal plasma: BSP A1/A2 (PDC-109), BSP A3 and BSP 30 kDa. The BSPs are secreted by the seminal vesicles (Magdaleno et al., 1997) and represent ~70% of the total protein content of seminal plasma (Nauc and Manjunath, 2000). They are single chain polypeptides with apparent molecular weights of 15000-16000 (PDC-109 and BSP A3) and 28000-30000 (BSP 30 kDa). The BSPs are small acidic proteins with a pI ranging from 3.6-5.2.

PDC-109 constitutes the most abundant protein of bovine seminal plasma [15-25 mg/ml] (Magdaleno et al., 1997; Scheit et al., 1988) and exists in a glycosylated (BSP A1) and non-glycosylated (BSP A2) form. The amino terminus of PDC-109 is 23 residues long and it has a single O-glycosylated residue at threonine 11 (Magdaleno et al., 1997). PDC-109 shares significant homology and analogy with HSP-1, a major
protein of stallion seminal plasma (Magdaleno et al., 1997) and with pB1, the boar seminal plasma protein ((Plucienniczak et al., 1999).

BSP A3 is comprised of 115 amino acids and is the only member of the BSP family which is not glycosylated (Manjunath et al., 1988). The BSPs share significant sequence homology but differ most significantly at the N-terminal domain. BSP 30 kDa is the most unique of this family as its amino terminal sequence shares no apparent similarity with other known protein sequences (Magdaleno et al., 1997). BSP 30kDa consists of 158 amino acids and has a unique 48-acid residue N-terminal extension which includes three 7-8- amino acid repeats and six O-glycosylated threononine residues (Magdaleno et al., 1997). BSP 30kDa contains a proline -rich region (residues 1-16) and three short amino acid sequence repeats (17-24, 25-31, 35-41) which make it unique among mammalian seminal plasma heparin-binding proteins.

The BSPs associate with epididymal sperm by binding to choline phospholipids (Desnoyers and Manjunath, 1992; Gerwig et al., 1996; Magdaleno et al., 1997; Manjunath et al., 1994a; Manjunath et al., 1994b; Scheit et al., 1988), specifically those in the outer leaflet of the plasma membrane. Ramakrishnan et al., 2001 demonstrated that PDC-109 exhibits the highest selectivity for the choline phospholipids phosphatidylcholine and sphingomyelin under physiologic pH and ionic strength, and that lipids which bear the phosphocholine moiety in the headgroup are clearly the lipids most strongly recognized by PDC-109. The presence of cholesterol in the membrane does not appear to affect the selectivity of PDC-109 for the choline-containing phospholipids, though the interaction of PDC-109 with different phospholipids is increased considerably in the presence of cholesterol (Swamy et al., 2002). The protein penetrates in to the hydrophobic interior of the membrane and interacts with the acyl chains up to the 14\textsuperscript{th} carbon atom (Muller et al., 1998).
The BSPs are comprised of two fibronectin type II domains in tandem, which are separated by a short linker polypeptide chain (Wah et al., 2002) and possess gelatin-binding properties (Romero et al., 1997). Each fibronectin type II domain contains a choline phospholipid binding site, and both of these sites are necessary for inducing lipid efflux (Desnoyers and Manjunath, 1992; Desnoyers and Manjunath, 1993; Esch et al., 1983; Moreau et al., 1998). The BSPs also contain heparin binding sites (Chandonnet et al., 1990; Desnoyers and Manjunath, 1992), and when bound to choline phospholipids, PDC-109 increases the heparin docking sites on the surface of sperm (Therien et al., 1995).

PDC-109 may mediate capacitation by stimulating cholesterol and phospholipid efflux from the plasma membrane (Manjunath and Therien, 2002; Therien et al., 1995) however, this only occurs in the presence of heparin or capacitating agents. Otherwise, PDC-109 increases the stability of the plasma membrane by reducing membrane fluidity and immobilizing cholesterol (Greube et al., 2001; Muller et al., 2002; Swamy et al., 2002) until glycosaminoglycans (GAGs) or high-density lipoproteins (HDLs) are encountered within the female reproductive tract. The degree to which PDC-109 causes immobilization of the membrane lipids increases as the degree of unsaturation likewise increases (Greube et al., 2001). PDC-109, itself, is lost from the plasma membrane during the process of capacitation (Therien et al., 2001), most probably occurring with the efflux of phospholipids. Calvete et al., 1994 reported that approximately 9.5 million PDC-109 molecules bind to the surface of a bull spermatozoon upon ejaculation. Following incubation in capacitating medium, the number of PDC-109 molecules is reduced to 7.7 million per spermatozoon. PDC-109 interacts with a number of ligands other than heparin, including collagen, fibrinogen, apolipoprotein (apo)A1, as well as apoA1 associated with high density lipoprotein (Chandonnet et al., 1990; Manjunath et al., 1989).
We have previously shown that PDC-109 (BSP A1/A2) promotes the binding of sperm to the epithelium that lines the oviduct (Gwathmey et al., 2003). Because PDC-109 shares such a great degree of structural homology with the remaining BSP proteins, BSP A3 and BSP 30 kDa, we sought to determine if these BSP proteins shared similar functions as PDC-109.

Binding of sperm to the oviductal epithelium prolongs survival. This has been demonstrated in a number of species (bovine - (Adams et al., 2000; Chian and Sirard, 1995); rabbit- (Smith and Nothnick, 1997); stallion- (Ellington et al., 1999b); human- (Ellington et al., 1999a; Pacey et al., 1995); Gwathmey et al., manuscript in preparation). The viability of sperm is prolonged by direct contact with the epithelium which serves to maintain motility by protecting the sperm from premature capacitation / acrosome reaction or other agents within the oviduct that cause chemical modifications to the sperm plasma membrane. Because epithelial binding has been shown to maintain motility, we reasoned that proteins which enable sperm to bind to the epithelium, may themselves, be responsible for the maintenance of motility by promoting epithelial binding. Therefore, we investigated the effects of PDC-109, BSP A3 and BSP 30kDa on sperm motility over time.

Finally, after evaluating the effects of the BSPs on sperm-epithelial binding and on maintenance of sperm motility, we assessed structural similarities and differences of the three proteins to lend insight to diverse and/or redundant protein function.

**Materials and Methods**

**Media and Chemicals**

Routine laboratory chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.
TALP (Tyrode Albumin Lactate Pyruvate) medium, a modified Tyrode balanced salt solution, was used for semen dilution, sperm incubation, oviductal explant preparation and incubation and consists of 99 mM NaCl, 3.1 mM KCl, 25 mM NaHCO₃, 0.39 mM NaH₂PO₄, 10 mM Heps, 2 mM CaCl₂, 1.1 mM MgCl₂, 25.4mM sodium lactate, 0.11 mg/ml sodium pyruvate, 6 mg/ml BSA (Fraction V, Calbiochem, La Jolla, CA), and 5 µg/ml gentamycin (pH 7.4, 290 mOsm).

Hepes Balanced Salts (HBS) solution was used for protein storage and dilution and was prepared by adding 25 mM Heps to 130 mM NaCl, 5 mM KCl, 0.36 mM NaH₂PO₄, 0.49 mM MgCl₂ and 2.4 mM CaCl₂ (pH 7.4, 290 mOsm).

Isolation and Purification of BSP Proteins

PDC-109 (BSP-A1/A2): PDC-109 was prepared as previously described (Gwathmey et al., 2003). Semen collected from bulls at Genex, CRI (Ithaca, NY) was transported to the laboratory at ambient temperature. Following supplementation with a serine and cysteine protease inhibitor cocktail (Complete™ EDTA-free, Roche Molecular Biochemicals, Indianapolis, IN), semen was centrifuged (3000 x g, 15 min) and filtrated (0.2 µm, cellulose acetate) to remove sperm and particulate debris, then assayed for protein content using the BioRad DC Protein Assay Kit (Hercules, CA). The seminal plasma obtained was stored at -20°C until use.

PDC-109 was isolated from seminal plasma according to the method of Gasset et al. (1997). Briefly, aliquots of seminal plasma containing 50-100 mg protein were applied to heparin-Sepharose CL-4B columns (1 x 20 cm) that were equilibrated with binding buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.4). After extensive washing, heparin-binding proteins were eluted with a 10 mM o-phosphorylcholine-containing binding buffer. The eluates were concentrated and dialyzed against 20 mM Tris-HCl (pH 6.5) in 1 M NaCl, then applied to DEAE-Sephadex equilibrated with the same buffer. Washings were repeated followed by
elution of PDC-109 with 10 mM o-phosphorylcholine in column buffer. Eluate fractions were pooled, concentrated, and dialyzed against PBS, water, or HBS, depending upon subsequent applications. Purity of PDC-109 was assessed by SDS-PAGE with silver staining (Blum et al., 1987).

**BSP A3 and BSP 30 kDa:** BSP A3 and BSP 30 kDa were prepared as described in Manjunath and Sairam (1987). Briefly, 100 mg of crude bovine seminal plasma was added to 15 ml of 0.05M sodium phosphate buffer (pH 7.1) and passed through a Mono-S column attached to a liquid chromatography system. The non-absorbed fraction was concentrated to 5 ml by ultra-filtration with a UM-2 membrane. The concentrate was then subjected to gel filtration. The purity of the proteins was assessed by SDS-PAGE according to Laemmli (1970).

**Preparation of Fresh Epididymal Sperm**

Fresh epididymal sperm were prepared as previously described (Gwathmey et al., 2003). Briefly, testes with attached epididymides and vasa deferentia were obtained from an abattoir (Taylor Packing Co., Wyalusing, PA) or from Genex CRI, Inc. (Ithaca, NY) and transported to the laboratory on ice. Epididymal sperm were flushed from the caudal epididymis via retrograde perfusion with TALP medium. The sperm obtained were washed twice by centrifugation in 5 ml TALP for 10 min (170 x g), and re-suspended in TALP at 5 x 10^6 cells/ml. Only samples with motility exceeding 85% were used.

**Preparation of Frozen-thawed Epididymal Sperm**

Straws containing epididymal sperm cryopreserved in egg yolk medium and stored in liquid nitrogen were submerged in a 37°C water bath for 35 s. The sperm suspension was then layered over a 45/90% discontinuous Percoll gradient and centrifuged for 15 min at 600 x g according to Parrish et al., 1995. After carefully
removing and discarding each layer of the gradient, the resultant dense layer of sperm was resuspended 5-fold in fresh Talp medium and washed for 10 min at 170 x g.

**Preparation of Ejaculated Sperm**

Bull semen was provided by Genex/CRI (Ithaca, NY), diluted five-fold in TALP immediately after collection, and transported to the laboratory in a 37°C warm water jacket. Within 60 min of collection, sperm were washed three times in 5 ml TALP (170 x g for 10 min), re-suspended in TALP at $5 \times 10^6$ cells/ml, and incubated at 39°C and 5% CO$_2$ in water-saturated air until assayed. Only samples with motility exceeding 85% were used. In designated experiments, frozen-thawed ejaculated sperm were used in lieu of fresh ejaculated sperm. These sperm were obtained prior to castration of the bulls to obtain paired ejaculated/epididymal samples.

**Preparation of oviductal explants**

Bovine oviducts were collected from a slaughterhouse (Taylor Packing, Wyalusing, PA), and transported on ice to the laboratory in PBS. Oviducts taken from cows in various cycle stages were used, as previous studies indicated cycle stage had no effect on sperm-epithelial binding (Lefebvre et al., 1995). Oviductal isthmi from a single cow were used in each experiment to reduce variation. Explants of oviductal epithelium were prepared as described previously (Lefebvre et al., 1997). Briefly, the isthmic portion of the oviduct was dissected free of connective tissue and rinsed in PBS. The epithelium was extruded in sheets by squeezing the oviduct with fine tweezers, then fragmented by pipetting, centrifuged for 1 min (170 x g), transferred to TALP, and allowed a minimum of 30 min incubation at 39°C, 5% CO$_2$ to form everted vesicles with apical, ciliated surfaces oriented outwardly. Explants were used within 6 h of slaughter.
**Sperm binding assays**

Binding assays were performed as described in Gwathmey *et al.*, 2003. Oviductal explants were centrifuged (170 g for 1 min in 5 ml TALP) and 10 µl of the pellet were added to 50 µl TALP. Ejaculated or epididymal sperm (5 x 10⁶ /ml) were then added in 20 µl aliquots. After 15 min incubation at 39ºC, 5% CO₂, loosely bound sperm were removed from explants by pipetting though three - 75 µl droplets of TALP. The explants were transferred to a four- cell Elisa plate (each treatment group in an individual cell) and covered with the accompanying lid.

Explants were videotaped on a 39ºC microscope stage using a Zeiss Axiovert Microscope (Carl Zeiss Inc., Thornbrook, NY). Videotaping was performed using a Dage CCD-72 black-and-white video camera (Dage-MTI, Inc. Michigan City, IN) in combination with a Panasonic AG- 7300 Super-VHS videocassette recorder (Panasonic Industrial Co., Secaucus, NJ) and a time/date recorder (For-A Corporation of America, Los Angeles, CA). At least 8 microscope fields of each treatment group were recorded and assessed for sperm binding density.

**Determining binding density**

In order to determine the density of sperm bound to explants, the video recordings were digitized and analyzed using an Apple - Talk Video Player (Apple Computers, Cupertino, CA). The numbers of bound sperm were determined using playback mode. Then, a video image of each explant was digitized to determine the surface area by employing National Institutes of Health (NIH) Image (internet-based free-ware at [http://rsb.info.nih.gov/nih-image/](http://rsb.info.nih.gov/nih-image/)). The binding density was calculated by determining the number of sperm bound per (0.1 mm)² of explant surface. Approximately 12.3 x 10⁴ ± 2.0 x 10⁴ mm² (mean ± SD) of explant surface were analyzed per treatment for each experiment performed.
Binding of epididymal sperm

Epididymal sperm were treated with either 15uM PDC-109, BSP A3, BSP 30 kDa or TALP diluent for 20 min at 39°C, then washed by centrifugation for 12 min (100 x g) to remove unbound PDC-109. The sperm pellet was re-suspended in TALP to a concentration of 5 x10^6 cells/ml and added to explants. Ejaculated sperm were used as a positive control for sperm binding. As described above, sperm / explant complexes were transferred though three TALP droplets to remove loosely bound sperm and placed under silicon oil for video-recording. The experiment was repeated four times, each time using sperm from a different bull and explants from a different cow.

Competitive inhibition of ejaculated sperm binding

To determine whether excess unbound BSP protein could competitively inhibit sperm binding to epithelium, washed ejaculated sperm were added to oviductal explants pre-treated with 100 µl of PDC-109 (250 µg/ml), BSP A3 (125 µg/ml), BSP 30kDa (62.5 µg/ml), to reflect the relative concentrations of protein found on the sperm surface, or buffer alone. Density of bound sperm was determined as described above. The experiment was repeated four times, each time using sperm from a different bull and explants from a different cow.

Effects of BSPs on sperm motility over time

Fresh epididymal sperm (10 x 10^6 / ml) were incubated with 15 uM PDC-109, BSP A3 or BSP 30 kDa for twenty minutes before addition to microtiter wells containing 40 µg/ml of bovine oviductal apical membrane vesicles. Video-recordings of sperm motility were obtained at 0, 1, 3, 5 and 8 hr of co-incubation. Treated sperm were incubated at 39°C and 5% CO₂ during assay. The percentage of progressively motile sperm was determined by reviewing recordings. At least 200 sperm were evaluated per treatment group.
Dose-dependent effect of BSPs on sperm motility

Frozen-thawed epididymal sperm were prepared as described above. 5μl aliquots of sperm suspension was added to 45 μl of oviductal apical membrane vesicles in Talp medium, incubated in microtiter wells at 39°C and 5% CO2 for 5 hr. Sperm were treated with 15 μg/ml, 7.5 μg/ml, 1500 ng/ml, 300 ng/ml, 60 ng/ml, 12 ng/ml or 2.4 ng/ml of PDC-109, BSP A3, BSP 30 kDa or HBS buffer. The motility of sperm was video-recorded at 5 hr and the progressive motility of each sample was determined. At least 200 sperm were assessed for each treatment group.

Preparation of oviductal membrane vesicles

Apical plasma membranes were isolated from the oviductal isthmus as performed in Boilard et al. (2002). Vesicles of apical plasma membranes derived from bovine isthmic oviductal epithelium were isolated from a total plasma membrane preparation by selective removal of basolateral membranes by Mg2+ precipitation.

Generating comparative structural models of BSPA3 and BSP 30kDa

The three BSP protein sequences were obtained from Genbank: PDC-109 (BSPA1/A2) (P02784), BSPA3 (NP_77625), and BSP-30kDa (P81019). Only the fibronectin type-2 (Fn2) domains of the BSP proteins were comparatively modeled as the N-terminal region of PDC-109 could not be solved because of the lack of electron density (Wah et al., 2002). PDC-109, BSPA3, and BSP 30kDa share a high degree of sequence similarity between each other (Salois et al., 1999), with the lowest level of sequence identity between PDC-109 and BSP-30kDa (55%). With 55% sequence identity, obtained from BLASTP, such alignments are sufficient for generating the comparative models. These amino acid alignments facilitated the generation of 3D theoretical models for each of the BSP proteins using the MODELLER6v2 program (Marti-Renom et al., 2000). The known structure of PDC-109 was used as a template on which to thread BSPA3 and BSP-30kDa. Upon generation of the two structural
models they were inspected visually to ensure the formation of the four conserved
disulfide bridges were intact. Each model was also quality-checked with the
“WHAT_CHECK” (Hooft et al., 1996) check program (http://www.cmbi.kun.nl/gv/se-
rvers/WIWWWI/), which found no major structural errors for either the BSPA3 or
BSP-30kDa models that were not already found in the template structure (pdbid =
1h8)(Wah et al., 2002). Electrostatic potentials and RMSD calculations were
performed using Swiss PDBviewer (Guex et al., 1999). The theoretical models have
been deposited into the models directory in the Protein Databank under protein
accession numbers XXXX-XXXX.

Statistical Analysis

All data are expressed as mean ± SEM. The data were analyzed using
ANOVA followed by Tukey HSD (honestly significant difference) pair-wise
comparison. Linear regression was applied to analyze dose-dependence.
STATISTIX® statistical software was used for analyses.

Results

In order to determine if BSP A3 and BSP 30 kDa could also enable binding of
sperm to oviductal epithelium, fresh epididymal sperm were coated with PDC-109,
BSP A3 or BSP 30kDa. Both BSP A3 and BSP 30 kDa significantly increased
binding above control values as observed with PDC-109 (Figure C.1). To verify that
the effects we observed with the BSPs were specific, we tested a small, acidic protein
of similar charge, myosin light chain (MLC). As shown in Figure C.2, the addition of
MLC to epididymal sperm did not significantly affect the binding of sperm to the
epithelium.

Previously we demonstrated that PDC-109 could effectively inhibit the binding
of ejaculated sperm to oviductal epithelium in a competitive and dose-dependent
Figure C.1
BSPs confer epithelial binding on epididymal sperm. Relative protein concentration present on sperm surface was used (4:1:1 of PDC-109: BSP A3: BSP 30 kDa). *Denotes significant differences among treatment groups. (Means ± SEM; N=4; P<0.01).
Figure C.2
Control for epididymal sperm binding to oviductal epithelium. MLC- myosin light chain. MLC, a small acidic protein, did not enhance binding of epididymal sperm.
*Denotes significant differences among treatment groups. (Means ± SEM; N=4; P<0.01).
No. sperm bound / (0.1 mm)^2 explant

CONTROL  PDC-109  MLC

* n=4; p<0.01
manner (Gwathmey et al., 2003). Likewise, pre-incubation of oviductal explants with BSP A3 or with BSP 30 kDa were equally effective as PDC-109 to significantly reduce binding density of ejaculated sperm (Figure C.3). The protein concentrations used in these experiments reflect the relative amount of proteins found on the surface of sperm, that is, a 4:1:1 ratio of PDC-109: BSP A3: BSP 30kDa; whereas the ratio of these proteins contained within seminal plasma is 10:1:1 (Nauc and Manjunath, 2000). These same experiments were conducted using equal protein concentration, yielding the same results (data not presented here).

Because it has been demonstrated that binding of sperm to oviductal epithelium prolongs the life span of sperm, and the BSPs effectively enable sperm to bind epithelium, we sought to determine if the maintenance of sperm motility was attributed to the action of the BSPs. Fresh ejaculated sperm that were treated with PDC-109, BSP A3 or BSP 30 kDa and co-incubated with oviductal apical membrane vesicles maintained a forward progressive motility significantly longer than untreated sperm co-incubated with the vesicles, or than untreated sperm alone. (Figure C.4). We also wanted to determine if this effect on maintenance of motility was dose-dependent. So we treated frozen-thawed epididymal sperm with various concentrations of BSP proteins, ranging from 2.4 ng/ml to 15 ug/ml, and incubated for 5 h before assessing the percent progressive motility. There was a positive correlation with the concentration of the BSPs and the % progressively motility of each treatment group, indicating the effect of the BSPs on motility was dose-dependent (Figure C.5). It also appeared that PDC-109 was more effective at maintaining progressive motility than was BSP A3 or BSP 30 kDa.

PDC-109 interacts with phosphatidylcholine (PC) and sphingomyelin, also to a lesser extent with phosphatidylglycerol (PG) and phosphatidylserine (PS) because of reduced affinity (Ramakrishnan et al., 2001). Binding of PDC-109 occurs in a single
Figure C.3
BSPs competitively inhibit ejaculated sperm binding. Control is standardized to 100%. *Denotes significant differences among treatment groups. (Means ± SEM; N=4; P<0.01).
*n=4; P<0.01
Figure C.4
BSPs prolong the forward progressive motility of fresh epididymal sperm co-incubated with oviductal membrane vesicles (OMV). (Means ± SEM; N=6; P<0.01).
Figure C.5
BSPs maintain the forward progressive motility of frozen-thawed epididymal sperm in a dose-dependent manner. % Motility was measured at 5hr co-incubation with OMV. The regression of dose on % motility was significant for PDC-109, BSP A3 and BSP 30 kDa (P< 0.01). Circles represent the mean ± SEM for six replicates.
step mechanism and the higher affinity of PDC-109 for PC occurs as a result of a faster association rate constant and a slower dissociation rate constant than with PE, PG or phosphatidic acid (Zdobnov et al., 2002). The presence of cholesterol in the membrane leads to an increase in the selectivity of PDC-109 for different phospholipids (Swamy et al., 2002). Binding of PDC-109 to PC does not appear to require calcium as other proteins do when binding to PC (Volanakis and Wirtz, 1979). Because of the kinetics of binding with PDC-109, little changes in protein conformation are required for binding to PC, while binding to other lipids such as PG or PA requires considerable changes in the conformation of the protein (Zdobnov et al., 2002), although Gasset et al., 2000 suggests that PDC-109 does undergo a conformational change upon binding to PC membranes. PC is traditionally thought to play merely a structural role in biological membranes and has not generally been observed to interact with integral or peripheral membrane proteins, as is the case with PDC-109 (Marsh and Horvath, 1998).

Though it has been previously demonstrated that PDC-109 has a greater binding affinity for lysophosphatidylcholine (LysoPC) than phosphorylcholine (PrC) (Desnoyers and Manjunath, 1992; Desnoyers and Manjunath, 1993; Magdaleno et al., 1997; Muller et al., 1998; Muller et al., 2002), Anbazhagan and Swamy (2005) have recently demonstrated that the binding of PDC-109 to LysoPC is 250-fold stronger than binding to PrC due to a small negative entropic contribution. Although they suggest that the binding of PDC-109 to PC is quite weak, when compared to LysoPC, they acknowledge that this interaction must occur, as well as binding to the acyl chains, for optimal interaction of PDC-109 with the sperm membrane. It seems that the glycerol backbone and the acyl chains in LysoPC are responsible for the increased binding affinity (i.e. interaction with the choline headgroups is weak and binding is optimized when interaction with the acyl chains is involved).
specificity of BSP 30 binding is much broader than the other BSPs; it binds to PE, PS, PI, phosphatidic acid and cardiolipin.

There is an optimal stoichiometric ratio of 12-15 phosphatidylcholine molecules per PDC-109 molecule. It is the density of phosphorylcholine and the surface charge that determine the interaction of the protein with the membrane since incorporation of PE or PS into PC vesicles reduces binding of PDC-109 (Muller et al., 1998).

Negatively charged residues (10 aspartic acids and 7 glutamic acids per PDC-109 molecule) correspond to 16% of the total amino acids of the protein (Esch et al., 1983). Most of the negative charges are concentrated within the N-terminal 23 amino acids. This polypeptide stretch contains 4 Asp and 6 Glu residues, suggesting the existence of a discrete and highly negatively charged domain within PDC.

BSPs potentiate capacitation of epididymal sperm exposed to heparin (Therien et al., 1995) and high density lipoproteins (HDL) (Therien et al., 1997). The BSPs actually bind to the sperm capacitation factors (Chandonnet et al., 1990)-heparin and HDL- (Manjunath et al., 1989). In order to do so, PDC-109 must form a dimer in order to actively function. PDC-109, which generally exists as a polydisperse aggregate in solution, is converted to a dimer upon binding of PC (Magdaleno et al., 1997). Heparin binding by PDC-109 is dependent on its aggregation state (Magdaleno et al., 1997), requiring oligomers of four PDC-109 molecules. Gasset et al., 1997 suggests that ionic and hydrophobic interactions are responsible for the aggregation tendency of PDC-109 monomers. Selective chemical modification indicates that 6 residues may participate in the heparin binding region of PDC-109: lys 34, arg 57, lys 59, arg 64, lys 68, and arg 104. Residues that are important for heparin binding are often remote in the amino acid sequence, but are often brought together in the folded protein. (Magdaleno et al., 1997). BSP 30 contains a polypeptide stretch (49-71) that
is homologous to type-A domains found in heparin-binding proteins from other mammalian species (Magdaleno et al., 1997). The C-terminal portion possesses the gelatin-binding fibronectin type-II module.

**Comparative structural modeling of BSPA3 and BSP 30kDa reveals sequence and structural features which support their similar functions to PDC-109.**

Similarities in function (described above) and sequence (Salois et al., 1999) of the three BSPs (PDC-109, BSPA3 and BSP 30kDa) suggests the protein structures of the BSPs may also be similar. The three-dimensional crystal structure of PDC-109 was determined previously by Calvete and co-workers (Wah et al., 2002). The structure consists of two tandem Fn2 domains. Each Fn2 domain of PDC-109 is able to bind a single phosphorylcholine molecule via a solvent exposed hydrophobic cluster consisting of three tyrosines (30/75, 54/100, and 60/108) and a tryptophan (58/106) (Figure C.6) (Salois et al., 1999). These two phosphorylcholine pockets also insert into the plasma membrane of sperm upon ejaculation (Muller et al., 1998). Choline phospholipids comprise over 70% of the total bull sperm (Watson, 1981) indicating a plethora of possible binding sites for PDC-109, BSPA3 and BSP-30kDa. Less is known about the residues found on the opposite face of PDC-109 which likely interact with the oviductal epithelium. PDC-109 is able to interact with heparin (Therien et al., 1997) which is thought to occur via interactions with two arginines (57, 64) and four lysines (59, 68, 85, 107), which form a patch of basic residues (Wah et al., 2002). Thus heparin-like glycosaminoglycans may be involved in the interaction of PDC-109 with the carbohydrate moieties responsible for its interaction with the oviductal epithelium.

The amino acid sequence identity between the three proteins’ Fn2 domains are: PDC-109 and BSPA3 (72%), PDC-109 and BSP-30kDa (55%), BSPA3 and
Figure C.6
Sequence features of the three BSP proteins: PDC-109, BSPA3, BSP-30kDa. Amino acid alignment of the three BSPs. Residues conserved in all three proteins are indicated with a * below the residue alignment. Aromatic residues which form the hydrophobic pocket to bind phosphorylcholine are underlined. Residues on the opposite face of the BSPs predicted to be important for docking of heparin-like molecules and interactions with the oviductal epithelia are in bold.
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PDC-109  MALQLGLFLIAGSVFVLQDVPVNGDQ---DEGVSTEP------------------------
BSPA3   MALRLGLFLIAGVSMQLDVPVNGDEQLSEIDVLPK--------------------------
BSP30   MAPLVLGLFLIAGAVFQQLHPVNGDIPDPGSKPTPPGMADELPTETYDPPEIYTTF
**  ********  *  ** **

PDC-109  --------------TQDGPAELPE---------DEECVFPPFVYRNKFDCTVHGSLPPW
BSPA3   --------------EKKDPASGAET--------KDNKCVFPPFIYNKYYFDCTLHGSLFLW
BSP30   LPRTIYPQEEMPYDDKPPSLSSKANDLNADVFGPCABFPFTYKGKYYMCTRNKNSVLLW
*  ***  *  ** ** *

PDC-109  CSLDADYVGRKFCQRTDYAKCVFPPFYGGKYETCTKIGSMWMS-WCSLSPNYDKRAW
BSPA3   CSLDADYFRKFCQTYKDYAKCVFPPFYEGKSYDTCTIIGSTFMNYWCSLSSNYLEDGVW
BSP30   CSLDTEYOGNKFCRTDEPFCVFPPFYRKCSSCFTLVHSSFFWRWCSSLTSNYDRDKAW
*  ***  *  ** ** *

PDC-109  KYC  183
BSPA3   KYC
BSP30   KYC
***
BSP-30kDa (57%). This sequence conservation along with function similarity, suggests the key residues involved in PDC-109’s binding to sperm and to the oviductal epithelium are also conserved in BSPA3 and BSP-30kDa and necessary for binding to both membranes. Indeed, tyrosines 30, 54, 75, 100, 108 and tryptophan 58 and 106 are conserved in both BSPA3 and BSP-30kDa (Figure C.6). However, residue 60 has a conservative change of a tyrosine to a phenylalanine in BSP-30kDa.

The residues involved in sperm binding thus are conserved among the three BSPs, however this is not the case for the residues on the opposite face of PCD-109 thought to be involved in oviductal epithelial binding. These predicted heparin binding residues (Arg57, Lys59, Arg64, Lys68, Lys85, and Lys107) are more diverged in BSPA3 and BSP-30kDa than the phosphorylcholine binding residues. These lysine and arginine residues have both conservative and radical amino acid changes in BSPA3 and BSP-30kDa (Figure C.6). This indicates the binding specificities of BSPA3 and BSP-30kDa to the oviductal epithelia may be slightly different.

In order to obtain a better understanding of the interactions of the BSPs key residues involved in membrane binding comparative structural models were generated for BSPA3 and BSP-30kDa based upon the known structure of PDC-109. The structural models generated for BSPA3 and BSP-30kDa both thread tightly to the PDC-109 structure (Figure C.7A). Only 0.42Å and 0.43Å RMSD separate the modeled structures from PDC-109 for BSPA3 and BSP-30kDa (352 atoms involved in each comparison), respectively. The residues comprising the phosphorylcholine binding pocket in BSPA3 and BSP-30kDa also form a hydrophobic by the aromatic rings which are present in a similar spatial orientation as their corresponding residues in PDC-109 (Figure C.7B). Thus, the binding of all three BSPs to sperm appears to occur via a similar mechanism; utilization of a hydrophobic pocket formed by aromatic residues within the Fn2 domain.
Figure C.7
Conserved structural features of the BSPs known (PDC-109) and modeled (BSPA3 and BSP-30kDa) structures. A: Superposition of PDC-109 (red), BSP-30kDa (blue), and BSPA3 (green) which show the secondary structural elements of the N-terminal Fn2 domain (left) and C-terminal Fn2 domain (right). An unbound phosphorylcholine is shown in the N-terminal Fn2 domain and a bound phosphorylcholine is shown in the C-terminal Fn2 domain (see arrows) (Wah et al., 2002). B: PDC-109 (red), BSP-30kDa (blue), BSPA3 (green) aromatic residues comprising the binding pocket is shown within a single Fn2 domain. A bound phosphorylcholine (orange) is shown surrounded by the four aromatic residues which form the hydrophobic pocket.
The residues which are suggested to be involved in interacting with heparin-like molecules are not as well conserved at the sequence level between the three BSPs as the phosphorylcholine binding sites. This suggests there may be differences in electrostatic or surface charge potential along the surface of the BSPs involved in oviductal epithelium binding. To determine whether any amino acid variability between BSPs at the oviductal epithelial interacting face change the binding specificity, their electrostatic surface potentials were examined. The most apparent differences are seen on the face of the BSPs, which interact with the sperm membrane. A strong positive electrostatic field is found in BSP-30kDa which is not found in BSPA3 or PDC-109, though both do have positive electrostatic fields within this region. This indicates there may be differences in the dynamics to which the different BSPs bind to sperm. On the opposite face only minor differences can be seen, the significance of which is not determinable.

**Discussion**

Epididymal bull sperm do not encounter the BSP proteins until ejaculation, when the proteins adsorb to the surface of the sperm. Therefore, epididymal sperm do not demonstrate a high affinity for epithelial binding as observed in ejaculated sperm (Gwathmey et al., 2003). These studies demonstrate that exposure of epididymal sperm to each of the BSP proteins increases the binding affinity for oviductal epithelium.

The first functional role for PDC-109 was suggested by Manjunath and co-workers (Therien et al., 1995) to promote heparin-induced capacitation of epididymal sperm which had been pre-incubated with the protein. This capacitation occurred as a result of lipid efflux from the sperm plasma membrane, stimulated by PDC-109. Yu et al., 2003 demonstrated that PKC activity is inhibited by PDC-109 and suggest that
the BSP proteins act as inhibitors of PKC activity, in vivo, to prevent premature acrosome reaction of sperm. Recently, Sanchez-Luengo et al., 2004 demonstrated that PDC-109 significantly increased the motility of sperm via activation of plasma membrane-bound calcium ATPases. Here, we demonstrate that PDC-109 as well as BSP A3 and BSP 30 kDa prolong sperm survival by enabling binding to epithelium to maintain motility.

It has been well-documented that sperm motility, viability, fertilizing capacity and hyperactivation are maintained when sperm are bound to epithelium of the female reproductive tract (reviewed in Suarez, 1998; Suarez, 2002) and that cultured oviductal epithelial cells (OEC) and their secretions have been shown to have a beneficial effect on motility of sperm (Eberspaecher et al., 1995; Ijaz et al., 1994; Richards et al., 2005). Boilard et al., 2002 showed a dose-dependent effect on maintenance of motility when ejaculated sperm were coincubated with oviductal apical plasma membrane vesicles (OAPM), most likely due to an increased number of binding sites available with an increase in OAPM quantity. With consideration of this information, we sought to determine the mechanism by which sperm motility and viability were maintained during epithelial binding. Previously, we identified and characterized a fucose-binding protein on bull sperm that binds spermatozoa to oviductal epithelium (Ignotz et al., 2001). We determined this fucose-binding protein was PDC-109 (BSP A1/A2), the predominant seminal plasma protein constituent and member of the BSP family of proteins. Therefore, we purified PDC-109 directly from seminal plasma and demonstrated its ability to promote sperm binding to oviductal epithelium (Gwathmey et al., 2003). Because of its similarities with the remaining BSP family members, BSP A3 and BSP 30 kDa, we sought to determine if these proteins could likewise promote sperm/epithelial binding. Our results show that each
of the BSP proteins can effectively mediate the binding of sperm to oviductal epithelium.

If, indeed, binding of sperm to epithelium prolongs motility/viability, and BSP proteins enable sperm to bind epithelium, then it would logically follow that BSP proteins may affect the motility of sperm. It has already been reported that BSPs alone do not prolong survival of epididymal sperm (Therien et al., 1995). However, we evaluated the motility effects of the BSPs with consideration of their function in mediating epithelial binding. With the introduction of OAPM in our motility assays, we determined that PDC-109, BSP A3 and BSP 30 kDa each prolonged the progressive motility of epididymal sperm. This prolongation of motility was a direct result of epithelial binding, as the motility of epididymal sperm co-incubated with OAMV in the absence of BSPs was significantly lowered. It has long been demonstrated that seminal plasma has detrimental effects on sperm motility and sperm storage due to components contained within (Shannon, 1965; Way et al., 2000). We suggest that the detriment of seminal plasma to sperm storage, in part, is due to the presence of excess, unbound BSP proteins which compete for binding to oviductal ligands to which sperm would attach to form a sperm reservoir, thereby maintaining motility and viability.

The results of our current investigation, taken together with published results from various other laboratories, indicate that each of the BSP proteins share common functions (e.g. stimulation of phospholipid efflux to effect capacitation, promotion of epithelial binding, and maintenance of sperm motility). Why does an apparent redundancy of protein function exist? If a protein performs an important function, it would seem plausible that some mechanism(s) would exist to ensure that this function is carried out; such as the binding of sperm to the oviductal epithelium during formation of the oviductal sperm reservoir that is necessary to ensure that sperm, in
the proper physiological state, are available to fertilize the egg to ensure propagation of the species. The redundancy in function may exist to make sure that the function is carried out even under a variety of circumstances or conditions. While each BSP is ‘able’ to carry out a specific function, such as binding, the primary role of that function in vivo, may differ. While all three BSP proteins are capable of enabling epididymal sperm to bind to the epithelium, competitively inhibit the binding of ejaculated sperm, and maintain the motility of sperm to prolong survival until fertilization occurs, perhaps one BSP family member primarily functions to confer epithelial binding upon sperm, while another functions primarily to competitively inhibit the binding of sperm, thus preventing ectopic formation of the sperm reservoir. Yet, another functions primarily to maintain sperm motility. While each of these proteins is capable of all of these functions, their main job may be to perform one function, but assist in the performance of the remaining functions under certain conditions, or should an imbalance in the production of the remaining BSPs occur. Isoenzymes, for example, are one redundant protein type commonly found in mammalian cells because the distribution of identical enzymatic activities between different subcellular compartments is a fundamental process of living cells. A variety of conditions may exist among female reproductive tracts. If ligands in the oviduct have evolved, perhaps the BSP proteins have evolved with such minor changes to accommodate binding and to alleviate barriers to fertilization among species or to avoid speciation. Proteins may form complexes with each other to potentiate the effects of one another to work synergistically, in vivo, which is common in cell interactions. When epididymal sperm are co-incubated with purified BSP proteins, they work synergistically (not additively) and in a concentration-dependent manner to induce capacitation, as measured by LPC-induced acrosome reaction (Therien et al., 1995).
Because of the apparent overlap in protein function of the BSPs, we examined their structural similarities and differences using threading software. This was possible by using the Modellar program and threading the sequences of the remaining BSPs over the known 3D structure of PDC-109 that was determined by crystallography (Wah et al., 2002). Generation of these 3D structures allowed us to make comparisons of the three BSP proteins and to observe differences that would otherwise not be readily apparent. Comparative structural modeling is useful in allowing us to observe differences in our proteins as well as to compare them to seminal plasma proteins that have evolved in other species.

We could use comparative modeling to evaluate function of proteins across species based on related structures and known sequences. Seminal plasma proteins from a myriad of other species have recently been determined to share heparin- and phosphorylcholine-binding properties with the BSP proteins of bovine: (bison [BiSV-16 kDa, BiSV-17 kDa, BiSV-18 kDa and BiSV-28 kDa] Boiservert et al., 2004, stallion-[HSP-1, HSP-2, HSP-12 kDa] (Magdaleno et al., 1997), boar-[pb1] (Magdaleno et al., 1997; Sanz et al., 1993), goat [GSP-14 kDa, GSP-15kDa, GSP-20kDa and GSP-22kDa] (Villemure et al., 2003), ram-[RSP-15 kDa, RSP-16 kDa, RSP-22 kDa and RSP-24 kDa] (Bergeron et al., 2005). These proteins are structurally related to the BSPs. Phosphorylcholine binding proteins from seminal plasma of many different species share antigenic determinants with the BSP proteins (including humans, hamsters, mice, rats and pigs.) (Leblond et al., 1993).

A rapid evolution of some seminal plasma proteins have been demonstrated (reviewed in Swanson and Vacquier, 2002). Therefore, it is not unreasonable to find three closely related proteins in bovine seminal plasma that share high sequence homology, but differ ever so slightly in their biological function (Mueller et al., 2004). Several laboratories, especially Manjunath and co-workers and Calvete and his
colleagues, have repetitively demonstrated the role of the BSP proteins in phospholipid efflux and resultant capacitation. Here, we present a novel functional role for the BSP proteins, providing protective effects to spermatozoa by enabling them to bind to oviductal epithelium, thus forming an oviductal sperm reservoir that not only prevents premature capacitation of sperm, but prolongs motility to ensure fertilization.