**The Mammalian Oviductal Sperm Reservoir: Identification of Receptors for Bull
Sperm on the Oviductal Epithelium**

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the Faculty of the Department of Biological Sciences
in Partial Fulfillment of the Requirements for a
Bachelor's Degree with Distinction in Research

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Abstract

In eutherian mammals, a storage reservoir is formed when sperm become trapped in the initial segment of the oviduct by binding to the epithelium lining its wall. In bull sperm, seminal vesicle secretory proteins in the bovine seminal plasma (BSP) protein family, namely PDC-109 (BSP A1/A2), BSP-A3, and BSP-30-kDa, coat the sperm head and enable sperm to bind to the oviductal epithelium. This study was undertaken to identify at least one receptor for BSP-30-kDa on the oviductal epithelium. Proteins extracted from apical plasma membranes of the oviductal epithelium were added to a column of BSP-30-kDa bound to anti-BSP-30-kDa covalently conjugated to Protein-A agarose beads. Unbound proteins were washed from the beads and proteins that bound to the column were eluted by adding EGTA, a calcium chelator, because sperm binding to the oviductal epithelium is dependent on calcium. The eluates were resolved on SDS PAGE gels, and silver staining detected two prominent bands of approximately 37 and 34 kDa. Tandem mass spectrometry identified the protein constituents of the bands as annexins (ANXA) 1, 2, and 4. Anti-annexin antibodies also labeled the cilia and apical surfaces of the epithelium in sections of bovine oviduct. Annexins belong to a multigene family of calcium-dependent, phospholipid binding proteins. Annexins are important in several biological processes such as cell adhesion, ion channel modulation, signal transduction, and regulation of membrane structure. It was concluded that annexins are primary candidates for the sperm receptors on bovine oviductal epithelium.

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Key Abbreviations

ANXA, Annexin
BSP, Bovine Seminal Plasma
BtLTL, Biotinylated *Lotus tetragonobolus* lectin
PBS, Phosphate Buffered Saline

Introduction

From an evolutionary perspective, a species' survival depends on its reproductive success. In cattle, the insemination of millions of spermatozoa into the female reproductive tract precedes the fertilization of usually only one oocyte. The odds against any one sperm reaching the oocyte are tremendous because sperm must overcome female immune responses and make their way deep into the female reproductive tract. Despite the seemingly hostile environment of the female reproductive tract, the female also protects the sperm that reach the oviduct by safely storing them in a reservoir until ovulation occurs. Sperm are trapped in the reservoir by binding to the oviductal epithelium, which maintains their survival during storage (Suarez et al., 1990). The sperm reservoir serves three functions. First, polyspermic fertilization is prevented because only a few sperm are released at a time to reach the oocyte in the oviductal ampulla (Polge et al., 1970; Hunter and Leglise, 1971). Second, the oviductal reservoir maintains sperm viability until ovulation. Third, the sperm's physiological state is regulated to ensure that the sperm are in the proper state when ovulation occurs (Chian et al.,1995).

The reservoir is formed when sperm become trapped in the initial segment of the oviduct by binding to the epithelium lining its wall (reviewed by Suarez, 2002). Sperm binding to the oviductal epithelium involves carbohydrate recognition, similar to the binding of a group of proteins called lectins to oligosaccharide ligands. In bull sperm, binding occurs via interactions between proteins on the sperm head and the glycoproteins on the oviductal epithelium. It has been shown that the presence of Ca^{2+} is required for binding of sperm to the carbohydrate ligand (Suarez et al., 1998). Fucose is the key

carbohydrate part of the ligands to which bovine sperm attach (Suarez et al., 1998). The bovine protein PDC-109 (Protein with N- terminus aspartic acid, D, and carboxy terminus Cystein, having 109 amino acids) has been shown to coat sperm and enhance sperm binding to the oviductal epithelium (Gwathmey et al., 2003). PDC-109 occurs in two forms, which include BSP-A1 and BSP-A2. BSP-A1 possesses a single trisaccharide, NeuNAc-Gal-GalNAc, that is *O*-linked via GalNAc to threonine residue number 11 (Calvete et al., 1999). BSP-A2, on the other hand, lacks the trisaccharide. PDC-109 is secreted by the seminal vesicles into seminal plasma and coats sperm by binding to choline phospholipids in the sperm plasma membrane (Ramakrishnan et al., 2001). Two other closely related seminal proteins, BSP-A3 and BSP-30-kDa, have also been shown to enhance sperm binding (Gwathmey et al., 2006). Each of the BSP proteins, including PDC-109, consists of a unique N-terminal domain followed by two tandem fibronectin type II domains that contain heparin and phospholipid binding sites (Chandonnet et al., 1990; Desnoyers and Manjunath, 1992). BSP- A3 consists of 115 amino acids and is the only non-glycosylated BSP (Manjunath et al., 1987). BSP 30-kDa is made up of 158 amino acids and has a unique 48-amino acid terminal sequence with three 7-8 amino acid repeats and six O-glycosylated threonine residues (Calvete et al., 1996). BSP 30-kDa contains a proline-rich region and three short amino acid sequence repeats that differentiate it from other mammalian seminal plasma heparin-binding proteins.

Bovine oviductal epithelial membranes contain a variety of fucosylated glycoproteins, but the ligand or ligands that interact with the BSP proteins have not yet

been identified. This study was undertaken to identify at least one ligand for BSP-30-kDa

on the oviductal epithelium, which is most likely a fucosylated glycoprotein.

Materials and Methods

Chemicals

Unless stated otherwise, chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Isolation and Purification of BSP-30-kDa

BSP-30-kDa was isolated and purified according to Manjunath and Sairam

(1987). Semen collected from bulls at Genex, CRI (Ithaca, NY) was transported to the

laboratory at ambient temperature. Seminal plasma was then prepared by adding a serine

and cysteine protease inhibitor cocktail (Complete[TM] EDTA-free, Roche Molecular

Biochemicals, Indianapolis, IN) followed by centrifugation (10,000 x g, 10 min) to

remove sperm. The supernatant was further clarified by passage through a 0.2 μm filter.

Seven volumes of cold (-20°C) ethanol were added with constant stirring to precipitate

proteins then held overnight at -20°C. The precipitate was collected by centrifugation,

washed in ethanol, centrifuged, and vacuum-dried.

The precipitated seminal plasma proteins (250 mg) were dissolved in 3 ml PBS

and applied to an Affi Gel 15 Gelatin 225 column (1.5 x 30 cm) (Bio-Rad Hercules, CA)

prepared according to Manjunath et al. (1987). The column was attached to an AKTA

FPLC (Pharmacia) at 4°C and equilibrated with PBS. After the proteins were allowed to

bind for 1 hr, the column was washed with PBS to remove unbound proteins. BSP

proteins were eluted from the column by using a gradient of 0 to 7 M urea in PBS.

PDC-109 and BSP-30-kDa co-elute at about 4-5 M urea (Manjunath et al., 1987).

Fractions (3 ml) were collected and evaluated for BSP content by SDS-PAGE gels

stained with silver nitrate (Blum et al., 1987) or colloidal Coomassie Blue G (Invitrogen,

Carlsbad, CA).

Fractions containing PDC-109 and BSP-30-kDa were pooled, placed in dialysis

tubing with a 3 kDa molecular mass cutoff, concentrated using polyethylene glycol (MW

20,000), and dialyzed against 20 mM Tris-HCl (pH 6.4) in 1 M NaCl. The resultant

fractions were then applied to a DEAE-Sephadex column equilibrated with the same

buffer containing 0.025% NaN_3. The pass-through material contained BSP-30-kDa,

which was concentrated and dialyzed against PBS at 4 °C. Purity of BSP-30-kDa was

assessed by SDS-PAGE according to Laemmli (1970).

Preparation of Apical Plasma Membrane Proteins from Oviductal Epithelium

Bovine oviducts were obtained from an abattoir (Cargill, Wyalusing, PA) and

transported in PBS on ice to the laboratory. The isthmic segment of the oviduct was

cleaned of mesosalpinx and rinsed in PBS. Epithelial sheets were extruded into cold PBS

supplemented with protease inhibitors (Complete[TM] EDTA-free, Roche Molecular

Biochemicals, Indianapolis, IN) by squeezing the segments with tweezers. The epithelial

sheets were washed twice by settling to remove blood cells. The sheets were collected by

centrifugation, and apical plasma membranes were prepared according to Murray and

Smith (1997). Cells were homogenized on ice in 60 mM mannitol, 5 mM EGTA, pH 7.4,

adjusted with Tris base and supplemented with protease inhibitors. The homogenate was

treated with 1 M $MgCl_2$ at 1/100 volume to precipitate non-apical membranes and stirred

for 30 min on ice. The homogenates were centrifuged at 3,000 x g to remove non-apical

membranes, and the supernatant fraction was re-centrifuged at 27,000 x g to recover

apical membranes. The pellet was resuspended in homogenization buffer and subjected to

another purification step of Mg^{2+} precipitation. Purified apical membranes were

resuspended in water and aliquots were stored at -80°C. Protein content of membrane

preparations were determined according to Lowry et al (1951).

Co-Immunoprecipitation

BSP-30-kDa bound to anti-BSP-30 kDa covalently linked to Protein A agarose

beads (Pierce Biotechnology, Rockford, IL) was used to isolate the ligands for BSP-30-

kDa as follows. Two ml of Protein A agarose beads were poured into a small

polypropylene column (0.8 x 4 cm). The column was washed and equilibrated with PBS

resulting in a final bed volume of 1 ml. Five to 10 mg of rabbit polyclonal anti-BSP-30-

kDa (provided by P. Manjunath) in 3 ml PBS was applied to the column. The column was

capped and placed on a rocking shaker for 1 hr at room temperature to allow antibody

binding. The column was washed with 30 volumes of PBS to remove unbound antibody.

The bound antibody was then covalently cross-linked to Protein A by adding to

the column 2 ml PBS containing 125 μl of 65 mM DSS (disuccinimidyl suberate) in

DMSO (Pierce Rockford, IL). The solution was mixed for 1 hr at room temperature to

facilitate cross-linking. The column was then drained and washed with 5 column volumes

of 50 mM glycine, pH 2.5 to stop the reaction and to remove non-covalently attached

IgG. After the column was washed with PBS, 200 μg of BSP-30-kDa was added to the column along with sufficient PBS to facilitate mixing. BSP-30-kDa was allowed to bind to the antibodies on the beads by placing the column on a rocking shaker for 4 hr at room temperature. The column was then washed with PBS to remove unbound BSP-30-kDa and equilibrated with BSA-free TALP medium (99 mM NaCl, 3.1 mM KCl, 25 mM NaHCO$_3$, 0.39 mM NaH$_2$PO$_4$, 10 mM Hepes, 2 mM CaCl$_2$, 1.1 mM MgCl$_2$, 25.4 mM sodium lactate, 0.11 mg/ml sodium pyruvate, and 5 μg/ml gentamycin pH 7.4, 290 mOsm).

Aliquots of oviductal apical membranes were solubilized by adding an equal volume of 2% octyl-β-glucopyrenoside (OBG) detergent in BSA-free TALP by gentle mixing for 1 hr at 4°C in the presence of protease inhibitors. A pre-clearing step had been performed to remove any membrane proteins that might bind nonspecifically to Protein A beads by adding the OBG-solubilized membranes to 100 μl of Protein A beads in TALP and incubating on a rocking platform for 1 hr at 4 °C. The beads were removed by centrifugation and the supernatant was loaded onto the column containing BSP-30-kDa bound to the antibody/Protein A beads. The column was incubated overnight with constant mixing at 4 °C.

Unbound proteins were removed by washing the column 5 times with BSA-free TALP, and bound materials were eluted with 5 mM EGTA because it had been demonstrated that sperm binding to oviductal epithelium is calcium-dependent (Suarez et al., 1998). Fractions of 0.5 ml were collected and prepared for PAGE using PAGE Prep Advanced Kit (Pierce) to concentrate proteins and remove interfering substances. Fractions were resolved on 12% SDS-PAGE gels and visualized by staining with silver

nitrate (Blum et. al., 1987) or colloidal Coomassie Blue G (Invitrogen, Carlsbad, CA).

This entire protocol was replicated at least two additional times.

Lectin and Western Blotting

     The gel obtained after SDS-PAGE was electroblotted onto a PVDF membrane

(Millipore Billerica, MA) according to Towbin et al (1979). The blot was then blocked

with 5% BSA in PBS with 0.05% Tween. The blot was probed with 20 ml of 1 μg/ml

Anguilla anguilla (AAA) fucose-specific lectin (EY Labs, San Mateo, CA) conjugated to

horseradish peroxidase (HRP) in TTBS solution (1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$) 0.05%

Tween in Tris-buffered saline and incubated for 1 hr. The blot was washed extensively

with TTBS. A solution for enhanced chemiluminescence detection (7.5 ml Supersignal

West Pico Luminol, 7.5 ml Stable Peroxide Solution) (Pierce Rockford, IL) was applied

to the blot for 5 min. The fluorescent protein bands on the blot were recorded on film for

chemiluminescence detection (Kodak) exposed to the blot from 2- 20 min. Another lectin

that was used for probing included biotinylated *Lotus tetragonobolus* lectin (btLTL, stock

1 mg/ml). The blot was stripped, blocked overnight with 5% PVA, and re-probed with

anti-annexin antibodies followed by goat-anti-mouse IgG conjugated with horseradish

peroxidase (GAM-HRP) at 1: 10,000 in TTBS. This protocol was replicated at least two

times with additional successful trials conducted by Dr. George Ignotz.

Mass Spectrometry

     The fractions obtained from the BSP-30-kDa/ anti-BSP-30-kDa column after

elution with 5 mM EGTA were pooled together to obtain enough protein for mass

spectrometry. SDS (225 μl) at a concentration of 0.5% was added to the pool. The pooled fractions were then centrifuged at 5,000 g for about 50 min at room temperature to concentrate the proteins. The proteins were then run on 10% SDS-PAGE gels and stained with Coomassie Blue. Protein bands of interest were cut out from the gel, placed into silicon-coated eppendorf tubes, and submitted for mass spectrometry (MS) at the Cornell Bioresource Center.

Protein Identification by Mass Spectrometry (protocol performed and provided by the Cornell Bioresource Center)

The protein bands were subjected to in-gel digestion by trypsin and tryptic peptide extraction following a protocol modified from Shevchenko et al. (1996). All gel-extracted supernatants were combined and evaporated to dryness by a Speedvac SC110 (Thermo Savant, Milford, MA).

The sample was reconstituted in 15 μl of 0.1% formic acid with 2% acetonitrile prior to (MS) analysis. The nanoLC was carried out by an LC Packings Ultimate integrated capillary HPLC system equipped with a Switchos valve switching unit (Dionex, Sunnyvale, CA). The gel-extracted peptides were injected using a Famous auto sampler onto a C18 μ-precolumn cartridge for on-line desalting and then separated on a PepMap C-18 RP nano column, eluted in a 30-minute gradient of 5% to 40% acetonitrile in 0.1% formic acid at 250 nl/min. The nanoLC was connected in-line to a hybrid triple quadrupole linear ion trap mass spectrometer, 4000 Q Trap from ABI/MDS Sciex (Framingham, MA) equipped with Micro Ion Spray Head ion source.

The data acquisition on the MS was performed using Analyst 1.4.1 software (Applied Biosystems) in the positive ion mode for information dependent acquisition (IDA) analysis. In IDA analysis, after each survey scan for m/z 400 to m/z 1550 and an enhanced resolution scan, the three highest intensity ions with multiple charge states were selected for tandem MS (MS/MS) with rolling collision energy applied for detected ions based on different charge states and m/z values.

The MS/MS data generated from nanoLC/ESI-based IDA analysis were submitted to ProID 1.4 search engine (Applied Biosystems) for database searching against the bovine database which was created as an interrogator search database into ProID 1.4. One trypsin missed cleavage, the carbobamidomethyl modification of cysteine, and a methionine oxidation were used for searching. The protein identification of annexins was based on the sequences of peptide fragments that had > 95% probability of matching with the particular annexin. The final protein identifications were reported using Pro Group Viewer 1.05 software (Applied Biosystems).

Immunohistochemistry

Animal care and use were approved by Cornell University, Ithaca, NY. Pubertal Holstein heifers aged 13-15 mo (D/B/A/ Faith Farm, Moravia, NY) were synchronized by 2 i.m. injections of 25 mg of prostaglandin F2α (5 mg/ml; Pharmacia and Upjohn, Kalamazoo, MI) according to and by Lefebvre et al. (1997). Oviducts were surgically recovered from estrous heifers, transported on ice to the laboratory in sterile PBS (pH 7.4), and washed in PBS. The isthmus was identified and separated by dissection into 1.0-cm segments and immersed in Bouin's fixative (1:10 volume ratio) for 22 hr. The tissue

was dehydrated, embedded in paraffin, cut into 8 to 10-μm sections, and mounted on poly-L-lysine-coated slides according to and by Lefebvre et al. (1997).

The tissue sections were deparaffinized in xylene for three 5 min intervals and rehydrated though graded dilutions of ethanol (100%, 95%, 70%, 30%) at 2 min intervals followed by a PBS wash for 5 min at room temperature. Antigen-retrieval was carried out by microwaving the slides in 600 ml of 10 mM Na citrate, pH 6.0 on high power for four 5 min intervals. Four 3 min H_2O washes and one PBS wash were carried out before treating the slides with 90% methanol with 3% hydrogen peroxide for 15 min at room temp to inactivate endogenous peroxidase activity. The sections were then washed with PBS for three 5 min intervals.

Sections were then blocked with normal goat serum for 30 min at room temp in a humid chamber. Primary antibodies: anti-bovine ANXA1 (mouse monoclonal, 1 mg/ml) (1:50 dilution in PBS + 10% normal goat serum), anti- bovine ANXA4 (mouse monoclonal, 1 mg/ml) (1:50 dilution), anti-bovine ANXA2 (mouse monoclonal, 1 mg/ml) (1:50 dilution) (BIOMOL, Exeter, UK), and anti-ANXA1 (mouse monoclonal, EH17a hybridoma supernatant) (Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA, 50 μg/ml, undiluted) were applied to separate sections and incubated overnight in a humid chamber at 4°C.

Secondary antibodies (goat-antimouse HRP at 1:50 dilution) were applied to the sections and incubated for 1 hr at room temp in a humid chamber. The tissue sections were then washed in PBS for four 10-min intervals. Staining was carried out using 5 ml of 100 mM Tris HCl, pH 7.6, 50 μl 1% H_2O_2, and 100 μl 3,3'-diaminobenzidine

tetrahychloride (DAB; Kirkegaard & Perry Laboratories, Gaithersburg, MD) by

incubating for 2-10 min at room temp. The reaction was stopped using H_2O, and

counterstaining of the tissue sections was done by applying 25 mg/ml of 2X Gill

hematoxylin (Fisher Scientific, Pittsburgh, PA) for 2 sec. Rinses with H_2O and PBS were

carried out before dehydrating the slides with ethanol and xylene. The slides were

mounted in Permount (Fisher Scientific) and visualized under a light microscope.

Negative controls included probing sections with mouse IgGs (1 mg/ml) of the

same dilutions as the primary antibodies. Sections were also labeled with secondary

antibody only or had only DAB applied as additional negative controls. This entire

immunohistochemical trial was replicated three times.

A lectin control was also included. Biotinylated *Lotus tetragonobolus* lectin

(btLTL, stock 1 mg/ml) was diluted in PBS and 1 mM divalent cation solutions of $CaCl_2$,

$MgCl_2$, and $MnCl_2$ to make dilutions of 1:1000, 1:200, and 1:100. For the control slide,

btLTL solutions of 1:500, 1:100, and 1:50 were diluted with equal volumes of 200 mM

fucose. The tissue sections were deparaffinized in xylene for three 5-min intervals and

rehydrated though graded dilutions of ethanol (100%, 95%, 70%, 30%) at 2 min intervals

followed by a PBS wash for 5 min at room temperature. The slides were treated with

90% methanol with 3% hydrogen peroxide for 15 min at room temp. The sections were

then washed with PBS for three 5-min intervals and blocked with normal goat serum for

30 min at room temp in a humid chamber. The btLTL and btLTL/fucose control solutions

were incubated with sections overnight in the humid chamber at 4°C. Streptavidin

peroxidase (stock 1mg/ml, dilutions 1:5,000 and 1:10,000) was then applied at room

temperature for 1 hr prior to staining with DAB and hematoxylin as described above.

Results

Oviductal proteins obtained from co-immunoprecipitation are identified as annexins

To identify its oviductal ligands, proteins extracted from apical plasma membranes of oviductal epithelium were added to a co-immunoprecipitation column of BSP-30-kDa bound to anti-BSP-30-kDa covalently conjugated to Protein-A agarose beads. Proteins that bound to the column were recovered by adding EGTA, a Ca^{2+} chelator because sperm binding to the oviductal epithelium requires Ca^{2+} (Suarez et al., 1998). When subjected to SDS PAGE, eluted fractions showed two bands of interest of approximately 37 and 34 kDa (Fig 1). All lanes showed bands for BSP-30-kDa as well as high- molecular weight proteins that were most likely unbound IgG's or keratins.
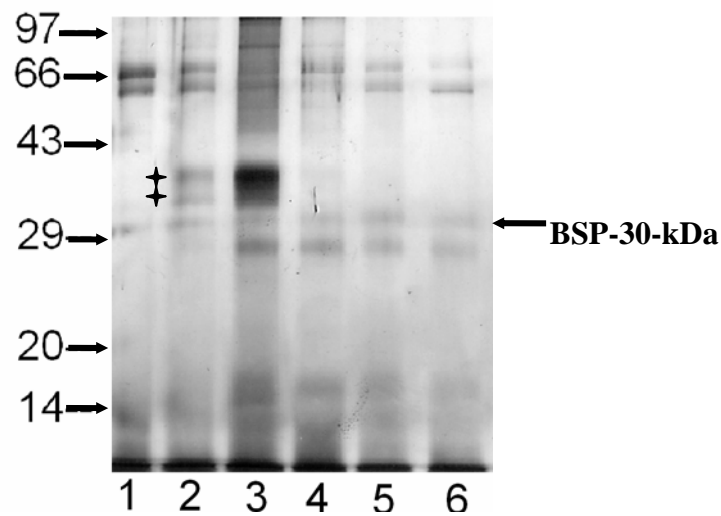
FIG. 1. SDS PAGE of fractions with molecular weights (kDa) indicated by arrows. Lane 1 indicates the last wash fraction, and lane 2 indicates the first fraction eluted with 5 mM EGTA. Asterisks indicate the two protein bands selected as candidate ligands for BSP-30-kDa, which were also observed in the two replicated experiments.

Mass spectrometry identified annexins 1 and 2 (ANXA1 and ANXA2) in the 37 kDa

protein band and annexin 4 (ANXA4) in the 34 kDa protein band (Table 1).

Table 1. Identification of BSP-binding proteins isolated from the protein bands selected in Fig. 1 for the species *Bos taurus* by mass spectrometry. (Table provided in part by Dr. George Ignotz.)

Protein Band[a]	Protein name	No. of peptides[b]	Sequence coverage[c]	Accession number[d]
37 kDa	ANXA1	13	38	gi|61553085
37 kDa	ANXA2	21	61	gi|59858385
34 kDa	ANXA4	10	34	gi|113956

[a]Protein Band selected in Fig. 1
[b]Number of peptides matching the sequence of the identified protein
[c]Percent of protein sequence covered by matching peptides
[d]NCBI accession number

Fractions obtained from at least two replicate experiments were electroblotted and probed with fucose lectin from Anguilla anguilla (AAA) lectin or biotinylated *Lotus tetragonobolus* lectin to determine whether the proteins contained any fucose, as would be predicted because fucose had been identified as a key component of the sperm receptor on the oviductal epithelium (Suarez et al., 1998). The lectins labeled the individual annexins obtained from the bands of interest (Fig. 2B). The fractions were also probed with anti-ANXAs to demonstrate the presence of annexins on apical membranes (Fig. 2A).
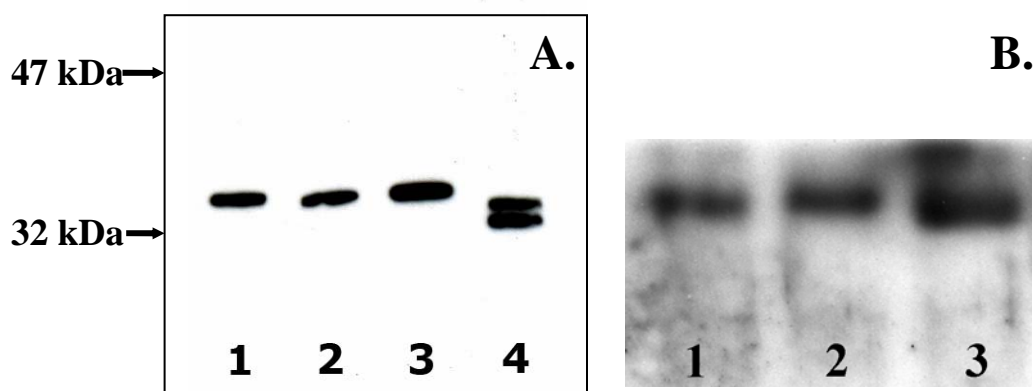

FIG. 2. Western blot detection and lectin probing of annexins in the oviduct epithelium. A) The PVDF membrane was probed with anti-ANXA1 (lane 1), anti-ANXA2 (lanes 2-3), and anti-ANXA4 (lane 4). Molecular weights (kDa) are indicated by arrows. B) ANXA1 (lane 1), ANXA2 (lane 2), and ANXA4 (lane 3) probed with lectin. (Figure provided by Dr. George Ignotz.)

Annexins are present on the surface of oviductal epithelium

ANXA1, ANXA2, and ANXA4 were localized by immunohistochemistry to the surface of epithelium in the bovine oviductal isthmus, the site of the sperm storage reservoir (Fig 3). All three annexins were localized on the apical surfaces and on cilia. Localization was patchy and the distribution of the staining differed among the three

annexins. Localization of fucose by LTL lectin was similar to that of the annexins; that is, staining was evident on the cilia and apical membranes of the epithelium (Fig. 3 I). None of the negative controls for antibody and lectin labeling were positively stained (Fig.3 B, D, F, H, J).
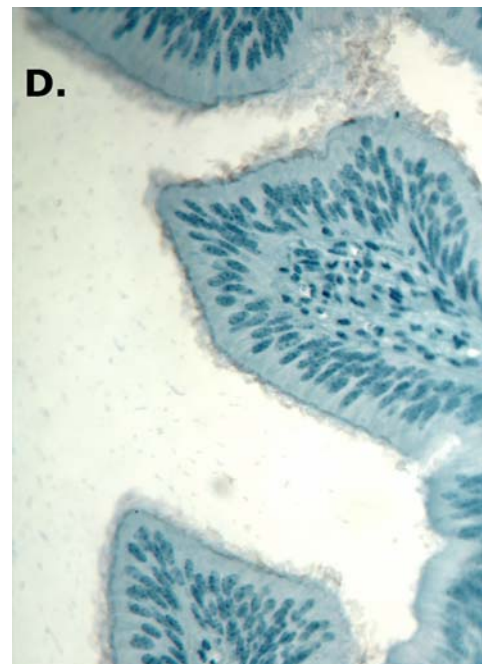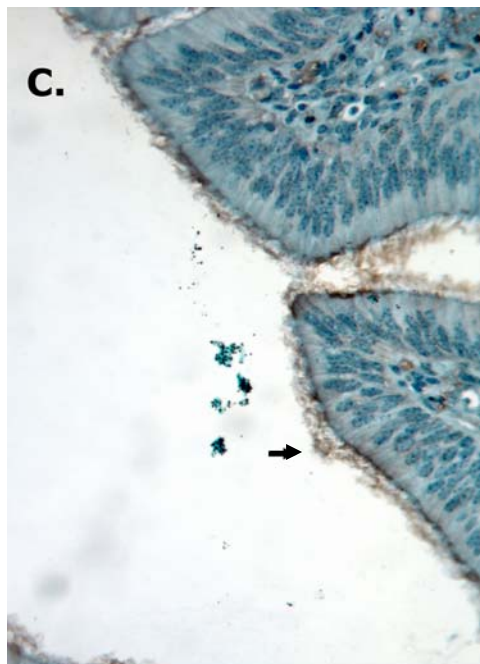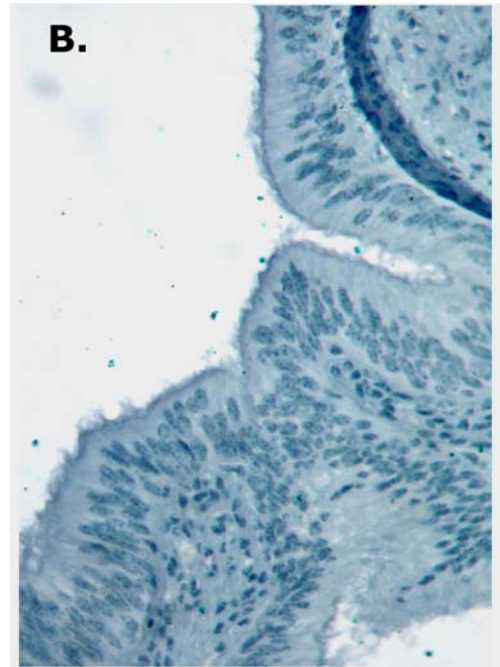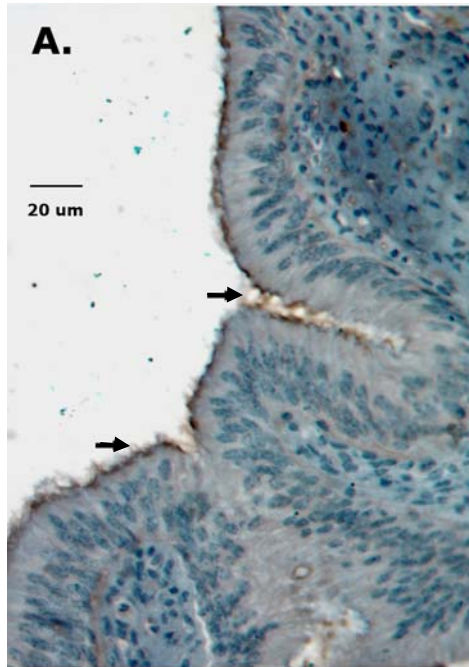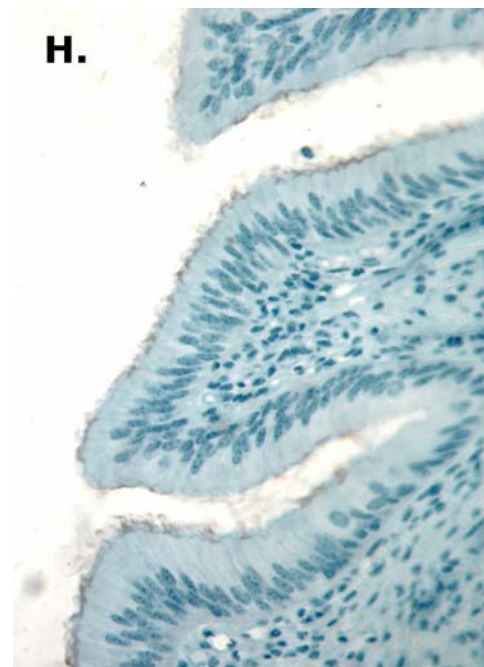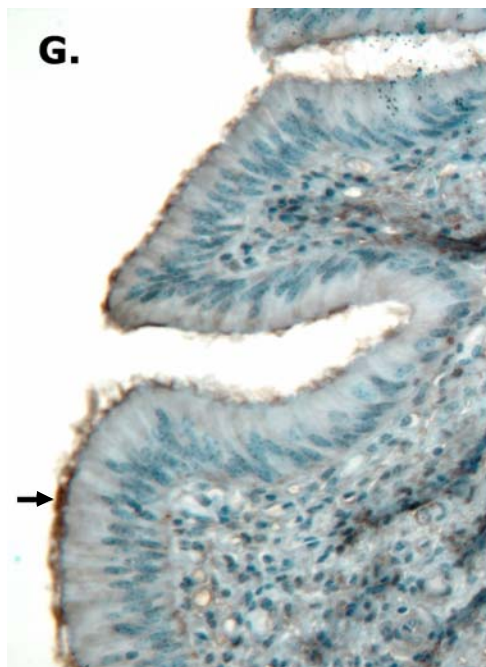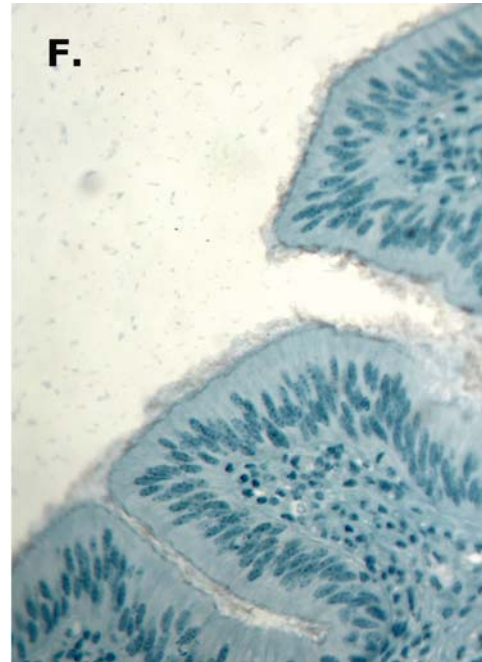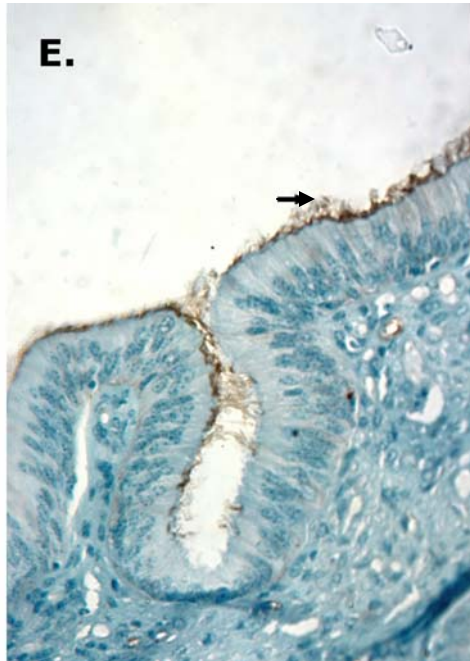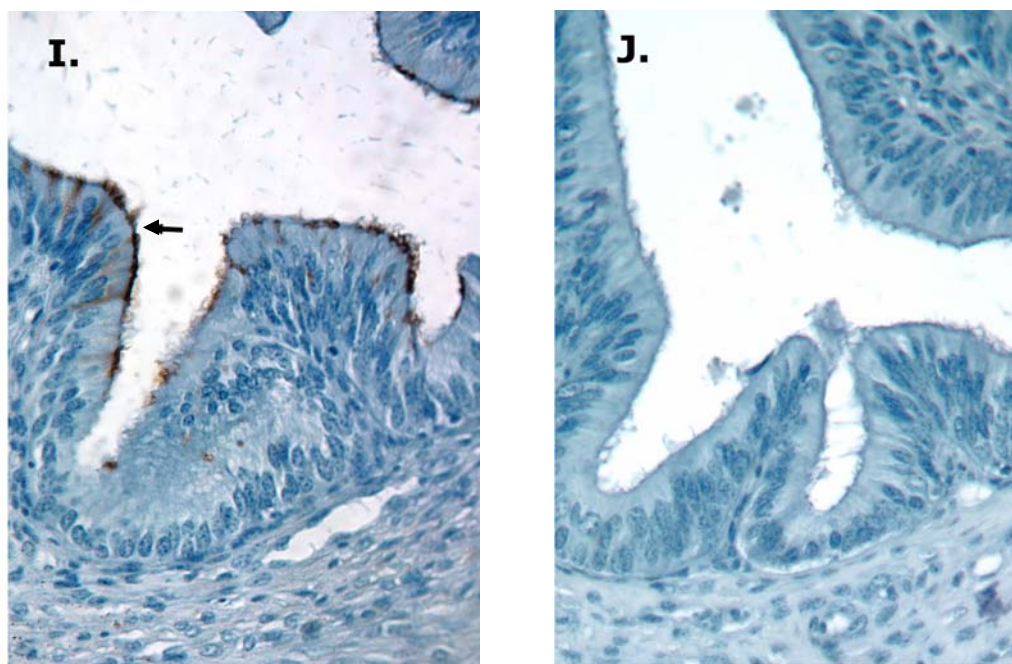
FIG.3. Immunolocalization and lectin localization of annexins in the bovine oviductal epithelium using light microscopy at the same magnification. Sections were probed with mouse monoclonal anti-ANXAs. Arrows indicate positive staining on the apical surface of the epithelium and cilia, which are sites for sperm attachment. A) anti-ANXA1 (EH17a); C) anti-ANXA1; E) anti-ANXA2; G) anti-ANXA4; Staining with the mouse IgG (controls: B, D, F, H) was negative. I) BtLTL: Patchy staining was evident on the cilia and apical surface, while the control containing fucose showed no staining (J).

Discussion

ANXA1, ANXA2, and ANXA4 were identified as receptors for BSP-30-kDa. These proteins bound to BSP-30-kDa/anti-BSP-30-kDa/Protein A columns and were confirmed to be present on the apical surfaces of oviductal epithelial cells by immunohistochemistry. Thus, annexins are likely candidates for receptors for sperm in the bovine oviductal sperm reservoir, which maintains sperm survival and viability until ovulation. Sperm binding to the oviductal epithelium involves carbohydrate recognition,

where terminally expressed fucosylated molecules are the key carbohydrate part of the

ligands to which bovine sperm attach (Suarez et al., 1998). BtLTL labeling of sections of

bovine oviducts confirmed the presence of fucose on the apical surfaces of epithelium.

Labeling of the annexin bands on the extracts of epithelium by the AAA fucose lectin and

btLTL confirmed the presence of fucose in the bovine annexins, whereas labeling by the

anti-ANXAs confirmed the presence of annexins on apical membranes.

Labeling of the epithelium by LTL fucose lectin and antibodies to ANXA 1, 2,

and 4 showed a patchy distribution.   This matches the pattern of sperm binding to

epithelium in vitro, which is also patchy (Lefebvre et al., 1995). It has been shown that

the presence of Ca^{2+} is required for binding of sperm to the oviductal epithelium and to

the fucose-containing trisaccharide Le^a (Suarez et al., 1998). The annexins were eluted

from the BSP-30-kDa/anti-BSP-30-kDa column by the addition of EGTA.

Taken together, our results indicate that the most likely candidates for the sperm

receptors are ANXA1, 2, and 4. These proteins belong to a multigene family of Ca^{2+}-

dependent, phospholipid binding proteins. Annexins have four homologous domains

(comprised of recognizable repeats with about 70 amino acids each) that are arranged in a

planar parallelogram (Siever et al., 1997). Annexins are important in several biological

processes such as cell adhesion, ion channel modulation, signal transduction, and

regulation of membrane structure (Siever et al., 1997). Furthermore, annexins are also

found in bovine endothelial cells (Gerke et al., 2002). Labeling of the endothelial cells

was apparent in some of the tissue sections used for immunolocalization of annexins in

the bovine oviductal epthelium and thus served as a positive control for the binding of the

anti-annexin antibodies.

So far, annexins are primary candidates that interact with BSP-30-kDa on the oviductal epithelium. It would be interesting to identify and compare the ligand(s) for all three closely related BSP proteins. Since the BSPs share high sequence homology, it is possible that all three BSPs bind to the same fucosylated glycoproteins. A final possibility to consider would be the role and significance of the differential distribution of the various annexins on the apical membranes of the epithelium. Further research will be needed to determine what other types of interactions exist among the BSP proteins and receptors found in the oviduct.

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