Sperm–Egg Recognition in the Mouse: Characterization of sp56, A Sperm Protein Having Specific Affinity for ZP3

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Abstract. Recognition between mammalian gametes occurs when the plasma membrane of the sperm head binds to the zona pellucida (ZP), an extracellular coat surrounding eggs. ZP3, one of three glycoproteins in the ZP, is the egg protein recognized by sperm. A mouse sperm surface protein, sp56 (M_r = 56,000), has been identified on the basis of its specific affinity for ZP3 (Bleil, J. D., and P. M. Wassarman. 1990. *Proc. Natl. Acad. Sci. USA.* 87:5563–5567). Studies presented here were designed to characterize mouse sperm sp56 and to further test whether or not this protein specifically recognizes ZP3. sp56 was purified by both ZP3 affinity chromatography and by ion exchange chromatography followed by size-exclusion chromatography. The purified native protein eluted from size-exclusion columns as a homomultimer (M_r ~110,000). Each monomer of the protein contains intramolecular disulfide bonds, consistent with its extracellular location. Immunohistochemical and immunoblotting studies, using monoclonal antibodies, demonstrated that sp56 is a peripheral membrane protein located on the outer surface of the sperm head plasma membrane, precisely where sperm bind ZP3. Results of crosslinking experiments demonstrated that the ZP3 oligosaccharide recognized by sperm has specific affinity for sp56. Collectively, these results suggest that sp56 may be the sperm protein responsible for sperm-egg recognition in the mouse.

THE mammalian egg is surrounded by the zona pellucida (ZP)[1], a porous extracellular glycoprotein coat ~7 μm thick that mediates species-specific sperm-egg recognition and the slow block to polyspermy. In mice, the ZP is composed of three glycoproteins called ZP1, ZP2, and ZP3 (Bleil and Wassarman, 1980*a*). The functions of all three ZP glycoproteins have been determined (Wassarman, 1988; Bleil, 1991).

Sperm-egg recognition is mediated by ZP3, a glycoprotein that has a polypeptide molecular mass of 44,000 D, and that bears both O- and N-linked oligosaccharides (Bleil and Wassarman, 1980*b*; reviewed by Wassarman, 1988). The part of ZP3 recognized by sperm is a ~3,900-D O-linked oligosaccharide (Florman et al., 1984; Florman and Wassarman, 1985; Bleil and Wassarman, 1988). This oligosaccharide will be referred to here as the "functional domain oligosaccharide" (FD oligo). Interaction of sperm plasma membrane with ZP3 involves tight "binding" between thousands of ZP3 molecules in the ZP- and ZP3-binding sites on sperm plasma membrane (Bleil and Wassarman, 1983; Bleil and Wassarman, 1986). Immediately after binding, ZP3 induces the acrosome reaction, a membrane fusion event in which plasma membrane of the sperm head fuses with the acrosome, a large lysozomal vesicle (Saling et al., 1979; Bleil Wassarman, 1983). Multiple point fusions result in the release of hybrid vesicles composed of sperm plasma and outer acrosomal membranes, and exposure of inner acrosomal membrane. Subsequent to the acrosome reaction, the sperm bores through the ZP, gaining access to and fusing with egg plasma membrane.

It has been proposed that "patching" of the sperm's ZP3 recognition protein on the sperm head leads to induction of the acrosome reaction (Wassarman et al., 1985; Leyton and Saling, 1989*a*; Bleil, 1991). According to the "patching hypothesis" (Bleil, 1991), ZP3, each polypeptide chain bearing two or more identical FD oligos, associates via these oligo-

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1. *Abbreviations used in this paper*: CAPS, (Na-[3-cyclohexylamino)-propane]-sulfonic acid; 2-D, two-dimensional; FD oligo, functional domain oligosaccharide; G-GAMIG, colloidal gold-bound goat anti-H+L mouse IgG; LG-GAMIG, colliodal gold-labeled goat anti-mouse IgG; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; PBS-BG, PBS containing 2 mg/ml BSA and 5% normal goat serum; TNBG, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mg/ml BSA, and 2% normal goat serum; TNMG, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.3% Tween, 0.5% Carnation instant milk, and 2% normal goat serum; ZP, zona pellucida.

saccharides with a lectinlike ZP3 recognition protein on sperm head plasma membrane: multiple, multivalent interactions between FD oligos and ZP3 recognition protein (binding) lead to aggregation of the sperm protein (patching) at the site of recognition between sperm plasma membrane and ZP. Patching of the ZP3 recognition protein triggers induction of the acrosome reaction in a manner analogous to other extracellular signaling systems, such as mast cell exocytosis.

We recently approached identification of the sperm's ZP3 recognition protein using ZP3 as a probe to tag or purify mouse sperm proteins having affinity for ZP3. Cross-linking and affinity chromatography studies identified a sperm protein with relative molecular mass of 56,000 that we called sp56. This protein is located on the extracellular portion of acrosome-intact sperm heads, is not present on acrosome-reacted sperm, and has specific affinity for ZP3 (Bleil and Wassarman, 1990). These properties are characteristic of the sperm protein responsible for recognition of ZP3.

Studies presented here have been carried out to further characterize mouse sperm sp56 and to test the hypothesis that sp56 is the sperm protein that recognizes ZP3 in mouse eggs. Native sp56 was found to exist as a homomultimer. The protein was shown to have specific affinity for ZP3's FD oligo. Finally, sp56 was shown to be a peripheral membrane protein located on plasma membrane overlying the sperm's acrosome, where ZP3-binding sites are located on the sperm.

Materials and Methods

Sperm Isolation

Retired breeder male mice (HSD:ICR strain; Harlan Sprague Dawley Inc., Indianapolis, IN), 8–18 wk of age, were killed and the cauda epididymes and vas deferens were transferred to prewarmed (37°C) M199M (Bleil and Wassarman, 1986) containing 5 mM EGTA. Tissue was sectioned with microscissors, and sperm were allowed to swim out for 10 min. Sperm were transferred to 9 vol of M199M and allowed to capacitate 30 min at 37°C. Capacitated sperm were purified by centrifugation at 25°C through a Percoll gradient at 5,000 rpm for 7 min in a Sorvall SS-34 rotor (Du Pont Instruments, Wilmington, DE). (The gradient had been prepared by centrifuging a 39-ml vol of 70% Percoll, 25 mM Tris-HCl, pH 7.4, 150 mM NaCl in a Sorvall SS-34 rotor for 1 h at 19,000 rpm.) Acrosome-intact and acrosome-reacted sperm were collected separately from the gradient as previously described (Bleil and Wassarman, 1990). Cells were diluted into 8 vol of medium and pelletted at 5,000 rpm in a table top centrifuge. The pellet was resuspended in buffer or medium for treatment in subsequent experiments. Sperm purified in this manner are fully motile and fertile. Capacitated sperm and capacitated, Percoll-purified sperm fertilized 8 out of 10 mouse eggs in a parallel experiment. An average of 4×10^7 purified sperm were isolated from each male. H_2O for all experiments was purified on a Nanopure system (Barnstead, Dubuque, IA).

Chromatography

Chromatographic separations of proteins were performed using an HRLC system (model 340; Bio Rad Laboratories, Richmond, CA) equipped with a dual wavelength UV/visible light detector, AST 386 computer, dot-matrix printer (linked to the computer), strip chart recorder (linked directly to the detector), and fraction collector. All chromatographic data are presented as reproductions of profiles from the strip chart recorder in unmodified form.

Two-dimensional SDS-PAGE

Protein samples were subjected to two-dimensional (2-D) SDS-PAGE (SDS-PAGE under nonreducing conditions in the first dimension, followed by SDS-PAGE under reducing conditions in the second dimension) as previously described (Bleil and Wassarman, 1980*a*), except that protein reduction was performed with 50 mM DTT. This method is useful for qualitative analysis of extracellular proteins. Extracellular proteins removed from the reducing environment of the cytoplasm can form inter- and/or intramolecular disulfide bonds. Those proteins migrate off the diagonal when subjected to 2-D SDS-PAGE. In all gel electrophoresis experiments, one lane of the gel contained SDS-denatured, DTT-reduced standards having apparent molecular masses of 200, 97.4, 69, 46, 30, 21.5, and 14.3 kD (Rainbow Standards; Amersham Corp., Arlington Heights, IL). Direct staining of gels was carried out by silver stain. Silver stain of 0.5–0.75-mm thick gels was performed as follows. Gels were fixed for 30 min in 10% acetic acid/30% isopropanol, and washed twice, 10 min each, in 10% isopropanol, followed by 5% isopropanol. Washed gels were reduced 10 min in 40 μM DTT and incubated 10 min in 0.1% silver nitrate. Gels were washed once in H_2O, twice in developer (50 μl 37% formaldehyde/100 ml 3% Na-carbonate), and developed in developer. Development was stopped in 1% acetic acid. Autoradiograms were prepared by exposing destained (Farmer's Reducer; Kodak Corp., Rochester, NY) dried gels to Kodak XAR x-ray film at −70°C.

sp56 Purification by ZP3-Affinity Chromatography

sp56 was purified by affinity chromatography essentially as previously described (Bleil and Wassarman, 1990). Percoll-purified, acrosome-intact sperm from 20 males were extracted in 2 ml buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM KCl, and 0.2% hydrogenated Triton X-100) containing 2 mg/ml turkey egg white trypsin inhibitor. (Under the conditions described here, UV-transparent, hydrogenated Triton X-100 (Calbiochem Corp., San Diego, CA) extracted 100% of sperm sp56, but only 12% of those sperm proteins solubilized by equimolar Triton X-100.) Insoluble material was removed by centrifugation at 14,000 *g* for 5 min. The sample was filtered through a 0.22-μm filter and loaded onto a ZP3 affinity column (Rainin EP column, 10 μM ZP3; Rainin Instruments, Woburn, MA) that had been constructed as previously described (Bleil and Wassarman, 1990). The column was washed with 20 vol of buffer A and bound protein eluted with a gradient of 0–8 M urea and 0.2% hydrogenated Triton X-100. Fractions (0.1 ml) were collected and analyzed by 2-D SDS-PAGE.

Photoaffinity Cross-linking of ZP3 Glycopeptides to Sperm

To identify ZP3 glycopeptides having specific affinity for sperm, pronase-generated glycopeptides from 2–4 μg ZP3 (purified as previously described, Bleil and Wassarman, 1986) were isolated, radiolabeled with ^{125}I-Bolton Hunter reagent (Amersham Corp.), and challenged with capacitated sperm as previously described (Florman et al., 1984; Florman and Wassarman, 1985). Sperm and bound glycopeptides were then extracted with 100 μl 1% SDS in H_2O. Insoluble sperm components were removed by microcentrifugation, the supernatant was diluted with 100 μl of 30% acetic acid, placed in ice for 20 min, and acid-insoluble material was removed by microcentrifugation. Approximately 90% of all sperm-associated counts were found in the acid-soluble supernatant. The supernatant was diluted with acetonitrile to a final concentration of 80% acetonitrile, 0.1% SDS, and 3% acetic acid, and brought to pH 5.4 with triethylamine. Radiolabeled glycopeptides prepared in this way were subjected to ion suppression chromatography, using an HPLC column (AX-5; Varian Analytical Instruments, Sunnyvale, CA) as previously described (Mellis and Baenziger, 1983). Glycopeptides separated by ion suppression chromatography elute as a function of oligosaccharide chain size and irrespective of peptide length. Estimated molecular weights were based on elution profiles of ^{125}I-labeled, pronase-generated glycopeptides from ovalbumin (Mellis and Baenziger, 1983). Fractions were collected and analyzed in a gamma counter and tested for activity as previously described (Bleil and Wassarman, 1988).

The sperm surface protein with which the putative FD oligo–bearing glycopeptide of ZP3 associates was identified by cross-linking sperm proteins to suitably modified ZP3 glycopeptides. Pronase-generated glycopeptides from 2 μg ZP3 were dissolved in 50 μl 0.1 M $NaHCO_3$, pH 8.5, and mixed with 5 μl 40 mM *N*-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA in DMSO; Pierce Chemical Co., Rockford, IL) for 1 h at 25°C in the dark. All subsequent steps, preceding photoactivation, were performed in a room illuminated with a red darkroom lamp. NHS-ASA–modified glycopeptides were isolated from the void volume of a BioGel P2 desalting column (in 0.1 M Na-phosphate, pH 7.5). Modified glycopeptides (300 μl) were iodinated by addition of ^{125}I-NaI (2 μl, 200 μCi in 0.1 M NaOH, ICN, Irvine, CA) and chloramine T (30 μl, 5 mg/ml in 0.1 M Na-

phosphate, pH 7.5). After 5 min, the reaction was stopped by addition of 30 μl 100 mg/ml Na-bisulfite in 0.1 M Na-phosphate, pH 7.2. After desalting over a BioGel P2 column in Dulbecco's PBS (GIBCO BRL, Gaithersburg, MD), radiolabeled glycopeptides (8×10^7 cpm) were challenged with 2×10^6 capacitated, Percoll-purified sperm in the same buffer containing 4 mg/ml polyvinyl pyrrolidone 40. Sperm were mixed by rolling at 25°C for 20 min and then diluted 10-fold into a quartz cuvette to a final volume of 5 ml in Dulbecco's PBS containing polyvinyl pyrrolidone 40. Photoactivation was performed immediately, using a mercury lamp (Carl Zeiss, Inc., Thornwood, NY) at full illumination at a distance of 8 cm for 30 s. Sperm were then washed three times by centrifugation in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, extracted with 1% SDS, and extracted protein (1,100–2,400 cpm total) subjected to 2-D SDS-PAGE. Gels were silver stained to identify the position of sp56, and autoradiograms of dried gels were developed.

Biochemical Purification of sp56

Percoll-purified, acrosome-intact sperm from 40–50 males were extracted with 2 ml 20 mM Tris-HCl, pH 8.5, 0.5% hydrogenated Triton X-100, and the extract was treated with 1 mM PMSF to inactivate proteases. The extract was centrifuged at 14,000 *g* for 5 min to remove insoluble material. The supernatant was filtered (0.22 μm filter) and loaded onto a 75 × 7.5-mm DEAE-5PW column (Bio Rad Laboratories). Protein was eluted with a 0–0.5 M NaCl gradient in column buffer (20 mM Tris-HCl, pH 8.5, 0.1% hydrogenated Triton X-100) over 20 min at 0.5 ml/min. Eluate was analyzed at 280 nm. 0.5-ml fractions were collected and subjected to 2-D SDS-PAGE, and the gels were silver stained to identify the position of sp56 (coordinates 40,000 D [unreduced], 56,000 D [reduced]). Fractions containing sp56 were pooled and concentrated on a filter (Centricon 30; Millipore Corp., Bedford, MA), and 200 μl were loaded onto a 300 × 7.8-mm BIO-SIL SEC 250 column (Bio Rad, Laboratories). The column was equilibrated and run at 0.2 ml/min with 50 mM Tris-HCl, pH 6.8, 0.1 M NaCl, 0.1% hydrogenated Triton X-100. Eluate was analyzed at 280 nm. 0.2-ml fractions were collected and subjected to 2-D SDS-PAGE to identify the position of sp56. Fractions containing sp56 were pooled, concentrated, and used for further studies. Molecular size determinations in the size-exclusion column were based on protein standards of >300, 150, 44, 17, and <10 kD. In cases where denatured proteins were separated (0.1% SDS replacing 0.1% Triton in the column buffer), standards of 200, 60, and 30 kD were used.

Amino Acid Sequencing

Protein samples containing ZP3 affinity-purified sp56, biochemically purified sp56, or total Triton-extracted protein from Percoll-purified mouse sperm were subjected to 2-D SDS-PAGE. Gels were equilibrated 15 min in transfer buffer (10% methanol, 1 μM DTT, 100 mM CAPS (Na-[3-(cyclohexylamino)-propane]-sulfonic acid), pH 10, and proteins were transferred to polyvinyldifluoride protein sequencing membrane (Bio Rad Laboratories) in that buffer at 4°C for 1 h at 300 mA in an electroblotting apparatus. The membrane was thoroughly washed in H_2O containing 1 μM DTT, and protein was detected by Ponceau stain containing 1 μM DTT. The stained protein located at the position corresponding to sp56 (40,000 D under nonreducing conditions and 56,000 D under reducing conditions) was sliced out of the membrane, thoroughly washed with 50% methanol and 50% H_2O containing 1 μM DTT, and either digested and extracted with trypsin or directly subjected to solid-phase amino acid sequencing. Tryptic fragments of sp56 separated by reverse-phase HPLC were isolated and subjected to solid-phase amino acid sequencing. Trypsin digestion, reverse-phase HPLC, and amino acid sequencing were performed by The Scripps Research Institute Protein Sequencing Core Facility.

Development of Anti-sp56 Monoclonal Antibodies

Mouse monoclonal antibodies, directed against sp56, were selected and cloned from hydridomas producing antibodies directed against total sperm proteins. Percoll-purified, acrosome-intact mouse sperm were separated into two groups. "Native" sperm were emulsified in 50% PBS (30 mM Na-phosphate, pH 7.2, 150 mM NaCl)/50% complete Freund's adjuvant. "Fixed" sperm were fixed 30 min (Z-fix; Anatech, Ltd., Battle Creek, MI). Z-fix, a formaldehyde solution containing zinc, was chosen as fixative because it has been shown to prevent formaldehyde-associated loss of native protein conformation and perforation of cell membranes (product information form, Anatech, Ltd.). Fixed sperm were washed twice by centrifugation in 0.1 M ammonium acetate, pH 9.0, and emulsified in 50% PBS/50% complete Freund's adjuvant. Each of five virgin female mice (28 d old) were injected intraperitoneally with 10^6 emulsified native sperm, and each of five additional animals were injected with 10^6 emulsified fixed sperm. Two boosts (10^6 fixed or native sperm emulsified in 50% PBS/50% incomplete Fruend's adjuvant) were given intraperitoneal by at 1-mo intervals, followed by a final intravenous injection (10^6 fixed or native sperm in saline). Spleens were dissected, fused with myeloma cell line P3X63AG8U.1, and hybridomas were grown and cloned by Synbiotics (San Diego, CA). Serial dilutions of antisera or hybridoma supernatants or monoclonal supernatants were tested for binding to purified sp56 by a solid-phase assay. 96-well plates containing biochemically purified sp56 (air dried, 4–6 ng/well) were "blocked" for 4 h, with rocking, in TNBG (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mg/ml bovine serum albumin, and 2% normal goat serum). (We routinely use this blocking procedure for all immunological studies to cover nonspecific antibody-binding sites and to cover Fc receptors of mouse sperm.) Antibodies were transferred to wells in TNBG and incubated overnight with rocking at 4°C. Wells were washed two times with TNBG. Colloidal gold–bound goat anti-H+L mouse IgG (G-GAMIG, 5 nm colloidal gold; Calbiochem Corp.) diluted 1:50 in TNBG was transferred to the wells and incubated with rocking for 4 hr at 25°C. Bound colloidal gold was detected by silver enhancement (Silver Enhancement Kit; Bio Rad Laboratories). (We have found that this solid-phase assay is ~10-fold more sensitive to comparable peroxidase- or alkaline phosphatase–dependent ELISA assays). Cloned cell lines secreting high affinity IgGs directed against plate-bound sp56 were grown as mouse ascites. Monoclonal antibodies were purified from ascites fluid by ammonium sulfate precipitation and DEAE ion exchange chromatography. Five monoclonal antibodies, designated 7C5 (IgG1, titer of 66 pM), 7H12 (IgG1, titer of 4 nM), 5F12 (IgG1, titer of 29 nM), 4B2 (IgG1, titer of 70 nM), and 12D2 (IgG3, titer of 400 nM) were selected for the current studies. All five monoclonal antibodies were derived from mice that had been injected with fixed sperm.

Western Blotting

Protein samples were subjected to either (*a*) SDS-PAGE under nonreducing conditions; (*b*) SDS-PAGE under reducing conditions; or (*c*) 2-D SDS-PAGE followed by transfer to an Immobilon P membrane (Millipore Corp.). After electrophoresis, gels (0.75-mm thick) were equilibrated 15 min in transfer buffer (10% methanol, 100 mM CAPS, pH 10), and protein was transferred at 300 mA for 1 h at 4°C or 20 mA overnight at 25°C. Membranes were washed and blocked by rocking for 4 h in TNMG (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.3% Tween, 0.5% Carnation instant milk, and 2% normal goat serum). Primary antibodies were exposed, with agitation, to membranes in TNMG for 16 h at 4°C. After three 2-min washes, membranes were incubated, with agitation, in G-GAMIG (diluted 1:50 in TNMG) for 4 h or in alkaline phosphatase–conjugated goat anti-mouse IgG (Calbiochem Corp.; 1:1,000 dilution in TNMG) for 1 h. Colloidal gold was detected by silver enhancement, and alkaline phosphatase was detected by BCIP/NPT Reagent (Calbiochem Corp.).

Immunoadsorption Experiments

Capacitated, Percoll-purified mouse sperm from one male were washed by centrifugation in 0.1 M Na-phosphate, pH 7.2, 1 mM EGTA. Pelletted sperm were resuspended and stored at −20°C in 1 ml 50% glycerol, 0.1 M Na-phosphate, pH 7.2. 100 μl of the suspension was extracted by addition of 0.1% Triton X-100 and 1 mM phenyl methyl sulfonyl fluoride. Insoluble material was removed by centrifugation in a microcentrifuge. Proteins were iodinated by addition of 200 μCi ^{125}I-NaI (2 μl in 0.1 M NaOH, ICN) and 10 μl 50 mg/ml chloramine T in 0.1 M Na-phosphate, pH 7.2, 0.1% Triton X-100. After a 5-min reaction, iodination was stopped by addition of 10 μl Na-metabisulfite (100 mg/ml in 0.1 M Na-phosphate, pH 7.2), and radiolabeled protein was purified over a BioGel P4 column equilibrated with 25 mM Tris-HCl, pH 7.4, 0.1% Triton. Radiolabeled protein (500 μl) was stored in this buffer containing 5 mg/ml bovine serum albumin at 4°C for a maximum of 4 d. Sperm proteins radiolabeled by this method had an average specific activity of 2×10^6 cpm/μg.

Immunoadsorption of radiolabeled sperm proteins was performed by binding protein to purified IgG that had been cross-linked to cyanogen bromide–activated Sepharose (Sigma Immunochemicals, St. Louis, MO). Aliquots (50 μl; 10^7 cpm) of radiolabeled sperm proteins were diluted into 200 μl Sandy's buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 0.1% SDS) containing 20 μl packed Sepharose (5 mg anti-sp56 antibody/ml packed Sepharose), and the sample was mixed 30 min. The suspension was washed three times in 2 ml Sandy's buffer, and most of the buffer was removed by centrifugation through a porous frit at 5,000 *g* for 1 min. Bound proteins were released from the Sepharose by cen-

trifugation of 65°C solubilization buffer (0.1 M Tris-HCl, pH 6.8, 2% SDS) through the bed. After subjecting samples to 2-D SDS-PAGE, gels were silver stained to determine the position of sp56, and autoradiograms of dried gels were developed.

Localization of sp56 on Mouse Sperm

Silver-enhanced immunogold staining was used to localize sp56 on sperm in the light microscope. Capacitated, Percoll-purified, acrosome-intact or acrosome-reacted mouse sperm were washed twice by centrifugation at 5,000 *g* in 50 mM Tris, pH 7.4, 150 mM NaCl. Washed cells were resuspended in the buffer and diluted fivefold in Z-fix (Anatech, Ltd.). After a 30-min fixation, the volume was diluted fivefold in 0.1 M ammonium acetate, pH 9.0, and the cells were washed twice by centrifugation in this buffer. Resuspended cells were allowed to air dry 10 min on charged glass slides (Fisher Scientific, Pittsburgh, PA). Sperm on glass slides were blocked with TNBG for 4 h at 25°C in a humidified chamber and exposed to anti-sp56 monoclonal antibodies or normal mouse IgG (40 μg/ml in TNBG) for 16 h at 4°C. Slides were washed twice with TNBG and exposed to G-GAMIG (1:40 dilution) for 4 h at 25°C. Slides were washed twice with TNBG, and colloidal gold was detected by silver enhancement. Arcosomal status of sperm was confirmed by Nomarski Differential Interference Contrast, as previously described (Bleil and Wassarman, 1986).

Immunogold staining was used to localize sp56 on replica plates of sperm in the electron microscope. Mouse sperm were fixed 1 h in Z-fix or paraformaldehyde and washed by centrifugation (3,000 *g*) in PBS. PBS-suspended sperm were allowed to attach without drying for 30 min to glass slides that had been treated with 3-aminopropyltriethoxysilane (Maddock and Jenkins, 1987). Attached sperm were blocked for 4 h at 25°C in a humidified chamber in PBS containing 2 mg/ml BSA and 5% normal goat serum (PBS-BG), then exposed to monoclonal antibody 7C5 or normal mouse IgG (100 μg/ml in PBS-BG) for 16 h at 4°C. Slides were washed twice with PBS-BG and incubated in PBS-BG containing 1:20 dilution of colloidal gold-labeled goat anti-mouse IgG (LG-GAMIG 30-nm diameter particles; BioCell, Cardiff, United Kingdam) for 4 h at 25°C. Slides were washed with PBS-BG, dehydrated through a graded ethanol series into isoamyl acetate, and dried with a critical point drying apparatus. Replicas of slide-bound sperm were made by shadowing at 60°C with pure platinum using a JFD-9000 shadowing apparatus (Jeol Ltd., Tokyo, Japan). Carbon backing was performed by rotary evaporation, and slides were submersed in sodium hypochlorite (domestic bleach) to dissolve tissue and remove replicas. Replicas were washed with H_2O, collected on carbon-coated copper grids (no. 200; Jeol Ltd.), and examined under an electron microscope (1200 EX; Jeol Ltd.).

Differential Extraction of sp56 from Mouse Sperm

Cauda epididymal sperm from one male mouse were washed by centrifugation in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl. The cellular pellet was resuspended in 200 μl of 150 mM NaCl, and 50-μl aliquots were diluted into 450 μl of each of four buffers: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl; 12.5 mM acetic acid (pH 3.5), 150 mM NaCl; 50 mM acetic acid (pH 3.0), 150 mM NaCl; or 200 mM acetic acid (pH 2.7), 150 mM NaCl. Resuspended cells were gently mixed by rotation for 30 min and pelletted by centrifugation. The presence of red blood cells in the cell population provided visible evidence that no buffer treatment resulted in extensive cell lysis. Supernatants at this stage evidenced no red color, and pellets contained visible, intact red blood cells. Supernatants containing proteins released from cells in buffer were removed, and 20% Triton X-100 was added to a final concentration of 0.5%. Cells were resuspended and extracted in their original buffers containing 0.5% Triton X-100. Triton-extracted sperm were pelletted by centrifugation, and supernatants containing Triton-extracted proteins that had not been released in test buffers were isolated. Both classes of proteins, those released from cells in buffer and those requiring detergent extraction to be solubilized, were subjected to Western blotting using anti-sp56 monoclonal antibodies as described above.

Results

Rationale

Purified ZP3, the egg protein recognized by sperm, was used in affinity chromatography and photoactivated cross-linking studies to identify mouse sperm proteins having specific affinity for ZP3 (Bleil and Wassarman, 1990). Those studies identified sp56 as the only sperm protein having specific affinity for ZP3. Studies performed here were designed to confirm and extend previous studies. Gel electrophoretic, chromatographic, and amino acid sequencing studies on purified sp56 were used to partially characterize the protein. Photoactivated cross-linking studies were used to determine whether ZP3's FD oligo evidenced specific affinity for sp56. Immunohistochemical studies were used to determine the location of sp56 on the sperm. Differential extraction of sperm was performed to determine whether p56 is a peripheral or integral membrane protein.

Affinity-purified sp56

Purification of sp56 by affinity chromatography using affinity columns bearing purified ZP3 was performed as described in Materials and Methods. Typical of many extracellular proteins, sp56, which elutes from a ZP3 affinity column under strongly denaturing conditions, apparently contains intramolecular disulfide bonds. This protein, subjected to 2-D SDS-PAGE, migrated as a "doublet," having coordinates of 40,000 D under nonreducing conditions and 56,000 D under reducing conditions (Fig. 1 *A*). The protein (~30 pmol) subjected to amino acid sequencing was found to have a single NH_2-terminal sequence, the first 12 amino acids of which are DXGPPPLLPFAS, where X is unknown.

An aliquot of the sample of Triton-extracted proteins from Percoll-purified, acrosome-intact sperm was subjected to 2-D SDS-PAGE. In such a gel (Fig. 1 *B*), protein comigrating with sp56 was a relatively faint doublet. Densitometric analysis of such gels suggests that sp56 represents only 0.2% of sperm proteins extracted with hydrogenated Triton X-100 (~2.3 pg or 25,000 mol sp56/sperm). Comparison of Fig. 1, *A* and *B*, suggests that sp56 from total Triton-extracted sperm proteins migrated to a unique position when subjected to 2-D SDS-PAGE. This conclusion is strengthened by the fact that both affinity-purified sp56 doublet (Fig. 1 *A*) and the doublet found at coordinates 40 kD (nonreduced), 56 kD (reduced) from total Triton-extracted sperm proteins (Fig. 1 *B*) had an identical NH_2-terminal amino acid sequence.

sp56, seen in Fig. 1 *B*, was undetectable on 2-D SDS-PAGE of Triton-extracted proteins from Percoll-purified, acrosome-reacted sperm (data not shown). This result agrees with previous observations, which showed that sp56 was absent from acrosome-reacted sperm (Bleil and Wassarman, 1990).

Crosslinking of ZP3's FD Oligo to sp56

Sperm recognize only a specific size class of ZP3 oligosaccharides (the 3,900-D O-linked FD oligo), not the protein's polypeptide chain (Florman et al., 1984; Florman and Wassarman, 1985; Bleil and Wassarman, 1988). Consequently, the mouse sperm's ZP3 recognition protein should have specific affinity for ZP3's FD oligo. Cross-linking studies were performed to identify sperm surface proteins that have affinity for glycopeptides bearing this oligosaccharide. Initial experiments were carried out to determine which size classes of ZP3 glycopeptides associate with mouse sperm. Pronase-generated glycopeptides of ZP3 were isolated, radiolabeled, and challenged with capacitated mouse sperm as previously described (Florman et al., 1984; Florman and Wassarman, 1985). Bound glycopeptides were then extracted and subjected to ion suppression chromatography to

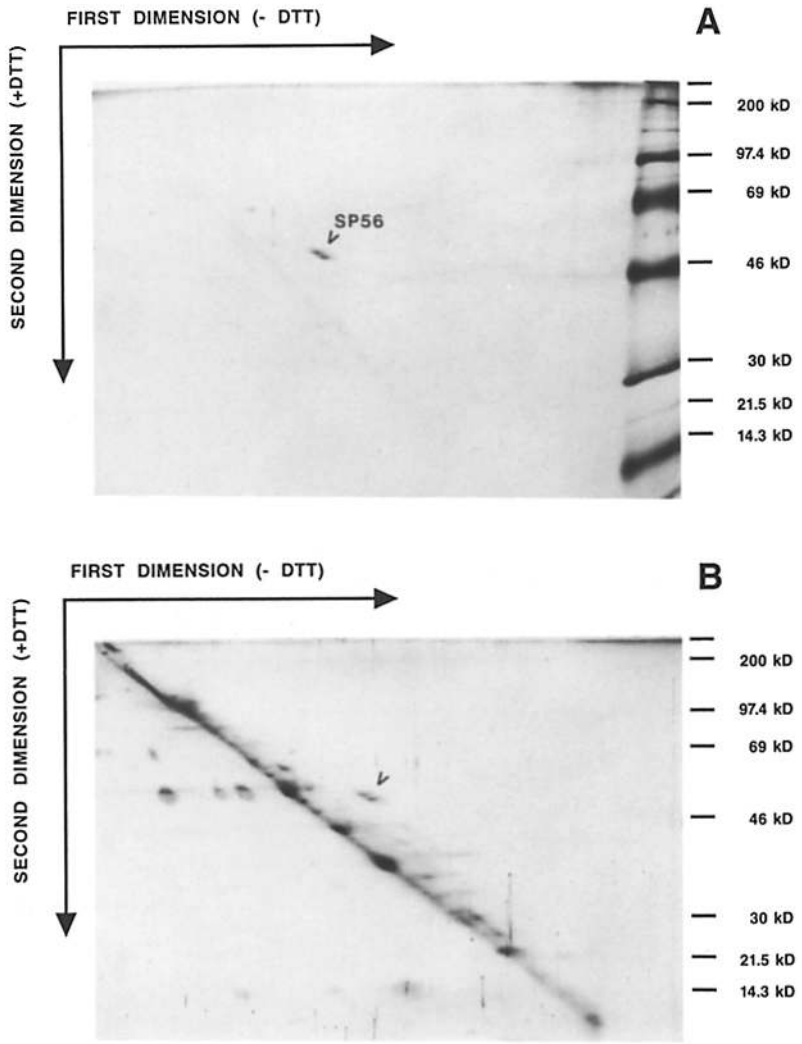


Figure 1. 2-D SDS-PAGE analysis of mouse sperm proteins and sp56 purified from a ZP3 affinity column. (*A*) Silver stained gel of affinity-purified sp56. Triton-soluble proteins extracted from Percoll-purified, acrosome-intact mouse sperm were subjected to ZP3 affinity chromatography as described in Materials and Methods. Protein eluted under denaturing conditions (6–8 M urea) was pooled and an aliquot was run on 2-D SDS-PAGE. sp56 migrated as a "doublet" above the diagonal of the second dimension gel. (*B*) Silver-stained gel of total Triton-extracted mouse sperm proteins. An aliquot of the sample of Triton-extracted sperm proteins was subjected to 2-D SDS PAGE before affinity chromatography. The position of sp56, having coordinates identical to sp56 in *A*, is marked with an arrow. Apparent molecular weights were estimated based on migration of molecular weight standards in a parallel well of the gel, as described in Materials and Methods.

separate glycopeptides on the basis of oligosaccharide chain size. Comparison of total radiolabeled ZP3 glycopeptides and radiolabeled ZP3 glycopeptides that associated with sperm during the challenge is shown in Fig. 2 *A*. These results demonstrated that only one size class of glycopeptides had specific affinity for mouse sperm, in agreement with previously published observations (Florman and Wassarman, 1985). The oligosaccharide chain size of the sperm-associated ZP3 glycopeptide was estimated to be ~3,900 D (range = 3,600–4,200 D), as previously shown for ZP3's FD oligo (Florman and Wassarman, 1985; Bleil and Wassarman, 1988). Competition assays, performed as previously described (Bleil and Wassarman, 1988), confirmed that only glycopeptides in the 3,600–4,200-D range blocked sperm-egg binding (data not shown).

To identify sperm surface proteins with which the putative FD oligo–bearing glycopeptide of ZP3 associates, ZP3 glycopeptides were modified with NHS-ASA and activated after challenge with capacitated mouse sperm. Total SDS-extracted sperm protein and protein-glycopeptide complexes were subjected to 2-D SDS-PAGE. An autoradiogram of such a gel (Fig. 2 *B*) demonstrates that the most heavily labeled protein comigrated with sp56. Spreading of radiolabel in the region of sp56 is likely caused by heterogeneity of the peptide and/or oligosaccharide chains of the glycopeptide. Radiolabel observed at the gel origin (>300,000 D in each dimension at the stacking/separating interphase) may represent cross-linked aggregates (see Discussion).

Biochemical Purification of sp56

Because of eventual clogging of ZP3 affinity columns, leaching of ZP3, and cost of column replacement, a biochemical method for purifying microgram amounts of sp56 was developed. Based on the observation that sp56 migrated to a unique position on 2-D SDS-PAGE (see above), chromatographic separations of mouse sperm proteins were analyzed by 2-D SDS-PAGE during the course of protein purification.

Triton-soluble proteins from Percoll-purified mouse sperm were subjected to DEAE ion exchange chromatography and fractions were analyzed by 2-D SDS-PAGE (Fig. 3 *A*). Fractions containing presumptive sp56 were pooled, concentrated, subjected to size-exclusion chromatography, and fractions were analyzed by 2-D SDS-PAGE (Fig. 3 *B*). The purified protein (Fig. 3 *C*) comigrated with affinity-purified sp56 on 2-D SDS gels. Two lines of evidence indi-

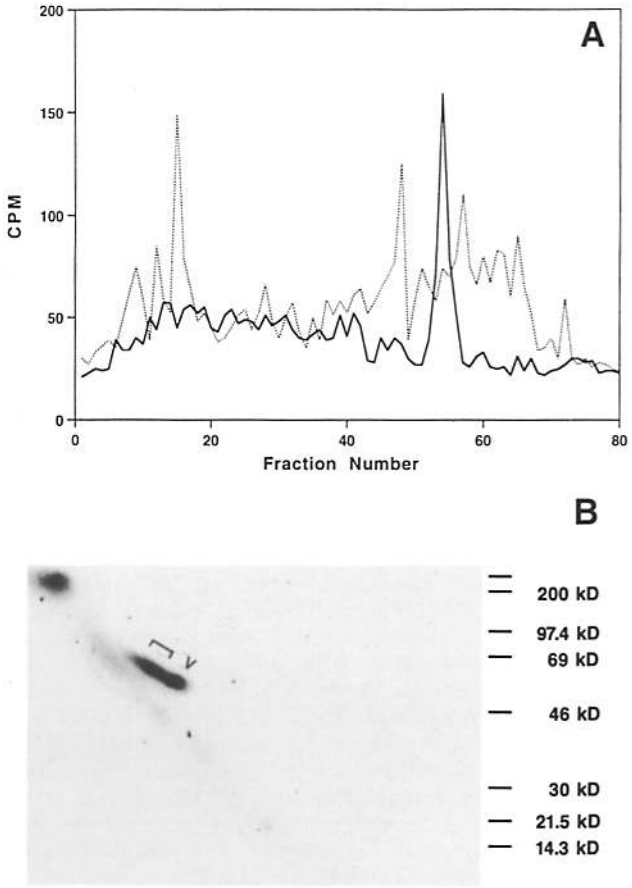


Figure 2. Association of putative FD oligo-bearing glycopeptide with mouse sperm and photoactivated cross-linking of the glycopeptide to mouse sperm proteins. (*A*) Ion suppression chromatography of total, radiolabeled glycopeptides derived from purified ZP3 (*dashed line*) and the fraction of those glycopeptides that bound to Percoll-purified, capacitated mouse sperm (*solid line*). The peak of sperm-bound, radiolabeled glycopeptides had an estimated oligosaccharide chain length of ~3,900 D. Procedures are described in Materials and Methods. (*B*) 2-D SDS-PAGE and autoradiographic analysis of sperm protein/glycopeptides formed by photoactivation. NHS-ASA-modified total glycopeptides were challenged with Percoll-purified, capacitated mouse sperm, and sperm-bound glycopeptides cross-linked to sperm surface proteins by photoactivation. SDS-extracted complexes were subjected to 2-D SDS-PAGE and an autoradiogram developed. Procedures are described in Materials and Methods. Radiolabel migrating in the region of sp56 had an estimated size range of 40–60 kD in the nonreducing dimension and 55–64 kD in the second dimension. The position of sp56 is indicated by an arrow. Radiolabel migrating close to, but not at, the position of sp56 is indicated by a bracket. Radiolabel migrating near the top of the gel (M_r >200,000) was located at the interphase between stacking and separating gels. Molecular mass estimations were as described in Fig. 1.

cate that affinity-purified sp56 and the biochemically purified protein are identical. First, the biochemically purified protein's NH_2 terminal amino acid sequence was identical to that of affinity-purified sp56 (see below). Second, monoclonal antibodies directed against the biochemically purified protein recognized both biochemically purified protein and affinity-purified sp56 (see below).

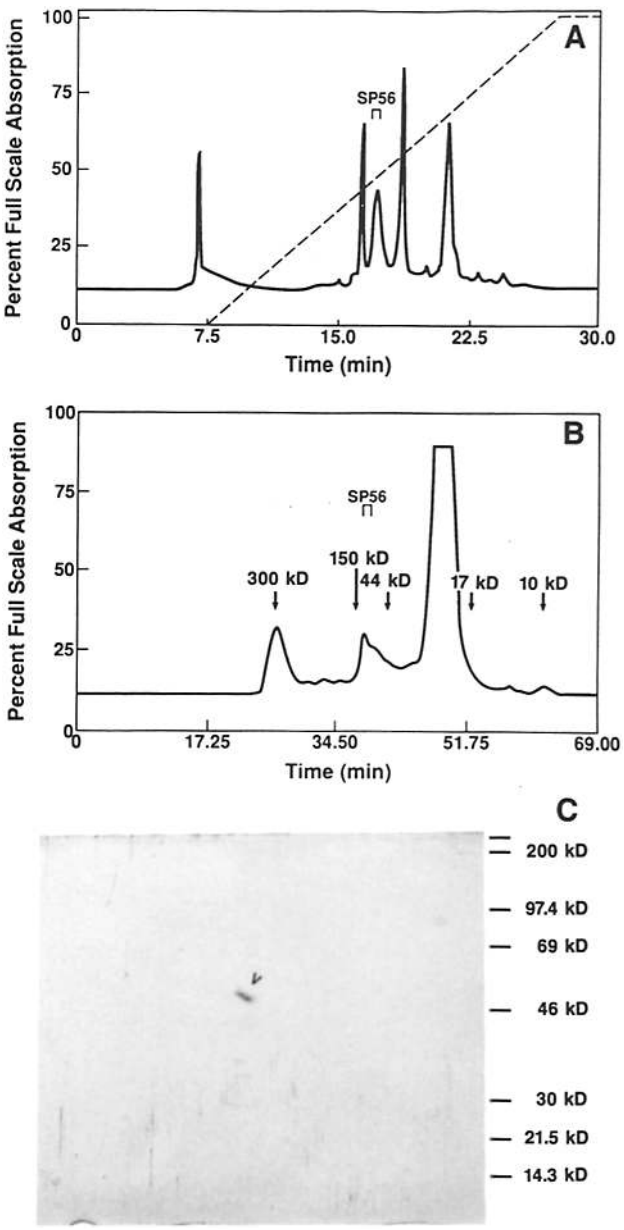


Figure 3. Biochemical purification of sp56. (*A*) Ion exchange chromatography. Triton-soluble proteins from Percoll-purified, acrosome intact sperm were subjected to DEAE ion exchange chromatography as described in Materials and Methods. Eluate was analyzed with a UV/VIS detector set to 0.5 absorption units full scale at 280 nm. The 0–0.5-M NaCl gradient is represented by a dashed line. Aliquots of fractions were subjected to 2-D SDS-PAGE. Fractions containing a protein comigrating with sp56 (*bracket*) were pooled and concentrated. (*B*) Size-exclusion chromatography. Pooled, concentrated fractions from the ion exchange column shown in (*A*) were subjected to size-exclusion chromatography as described in Materials and Methods. Eluate was analyzed with the detector set to 0.1 AUFS at 280 nm. The positions of size-exclusion standards, run in the same column, are designated. Aliquots of fractions were subjected to 2-D SDS-PAGE. Fractions containing a protein comigrating with sp56 (*bracket*) were pooled and concentrated. (*C*) Silver-stained SDS-PAGE of an aliquot from the pooled, concentrated fractions from the size-exclusion column shown in (*B*). The position of sp56 is marked with an arrow. Molecular mass estimations were as described in Fig. 1.

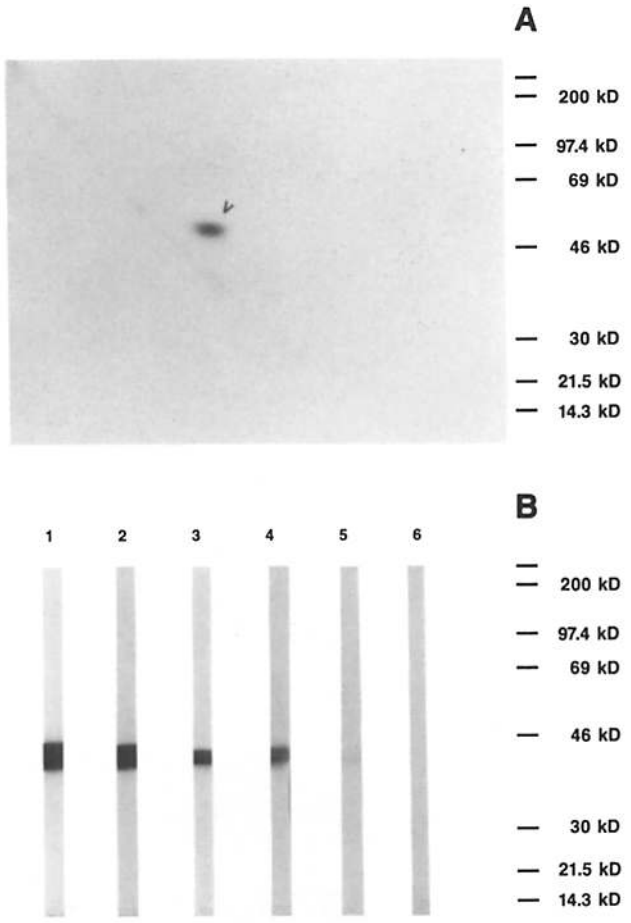


Figure 4. Immunoadsorption and Western blotting analysis of monoclonal antibodies directed against biochemically purified sp56. (*A*) 2-D SDS-PAGE autoradiogram of ^{125}I-labeled sperm proteins immunoadsorbed with monoclonal antibody 7C5. ^{125}I-labeled proteins from Percoll-purified, Triton-extracted sperm were immunoadsorbed using 7C5-Sepharose as described in Materials and Methods. Immunoadsorbed protein was subjected to 2-D SDS-PAGE, the gel stained, and an autoradiogram was developed. The position of sp56 is marked with an arrow. (*B*) Western blot analysis of sperm proteins recognized by five anti-sp56 monoclonal antibodies. Triton-extracted proteins from Percoll-purified sperm (in a wide lane, nonreduced) and prestained protein standards (in a thin lane, reduced) were subjected to 1-D SDS-PAGE and electroblotted onto an Immobilon P membrane as described in Materials and Methods. Strips were cut from the membrane, exposed to antibody (40 μg/ml), and bound antibody detected by silver-enhanced G-GAMIG staining, as described in Materials and Methods. Lanes *1–5* were exposed to monoclonal antibodies 7C5, 7H12, 5F12, 4B2, and 12D2, respectively. Lane *6* was exposed to normal mouse IgG. Each monoclonal antibody bound to a doublet corresponding to the position of nonreduced sp56 (M_r ~40,000). Variations in intensity reflect relative titers of antibodies (see Materials and Methods).

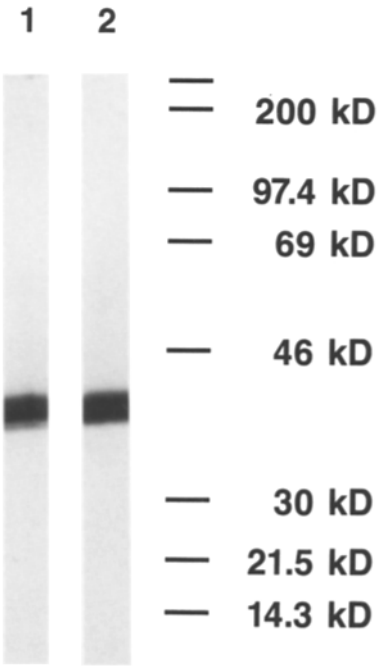


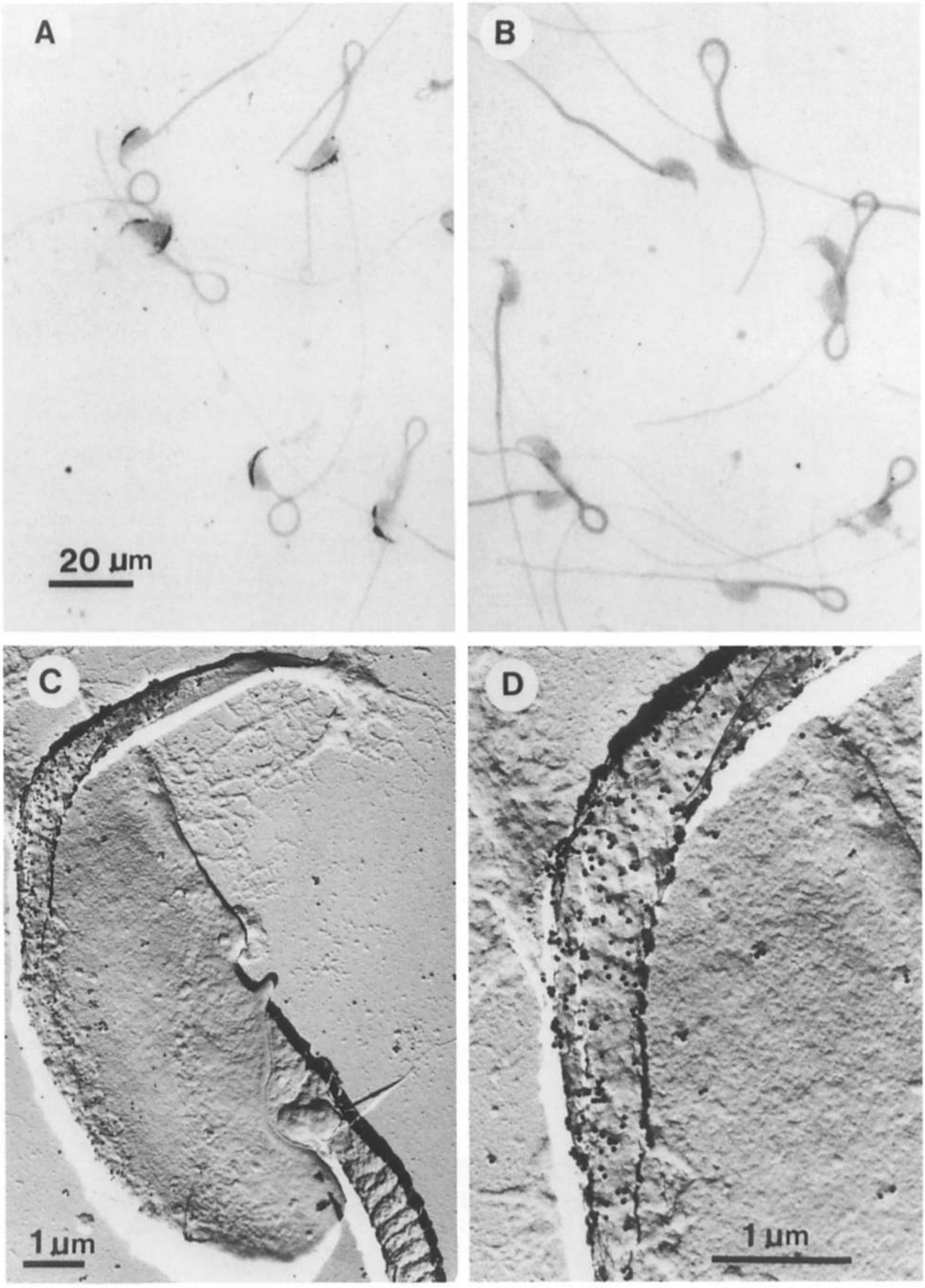
Figure 5. Western blot analysis of affinity-purified and biochemically purified sp56. Affinity purified sp56 (lane *1*) and biochemically purified sp56 (lane *2*) were subjected to 1-D SDS-PAGE under nonreducing conditions in parallel with a lane containing reduced, prestained standards as described in Materials and Methods. Proteins were electroblotted onto an Immobilon P membrane, the membrane was exposed to monoclonal antibody 7C5 (40 μg/ml), and bound antibody was detected by silver-enhanced G-GAMIG staining, as described in Materials and Methods. Both proteins, recognized by monoclonal antibody, migrated as a doublet in the position of nonreduced sp56 (M_r ~40,000).

Biochemically purified sp56 eluted from size-exclusion columns at an estimated molecular mass of 110,000 D (range = 90,000–130,000 D, based on size standards: Fig. 3 *B*). This elution profile does not appear to be an artifact of aggregation during purification. When total Triton-extracted sperm proteins were subjected to size-exclusion chromatography under nondenaturing (0.1% Triton) conditions, fractions containing sp56 (determined by 2-D SDS-PAGE profile) eluted at 110,000 D. When sperm proteins were subjected to size exclusion chromatography under denaturing (0.1% SDS) conditions, fractions containing sp56 (determined by 2-D SDS-PAGE profile) eluted at 46,000 D (data not shown).

Biochemically purified sp56 (~80–100 pmol) subjected to amino acid sequencing had an NH_2-terminal amino acid sequence, the first 12 amino acids of which are DCGPPPLLPFAS. Using relatively large amounts of biochemically purified sp56 (compared to amounts obtained by affinity chromatography) allowed assignment of amino acid no. 2 as cysteine. One trypsin fragment of the protein gave an amino acid sequence of (G/S)YLAPGF, where (G/S) indicates either G or S in this protein.

Anti-sp56 Monoclonal Antibodies

Five monoclonal antibodies, 7C5, 7H12, 5F12, 4B2, and 12D2, were selected on the basis of their recognition of plate-bound, biochemically purified sp56. Immunoadsorption experiments, using total ^{125}I-labeled Triton-extracted proteins from Percoll-purified mouse sperm demonstrated


A
20 µm
B
C
1 µm
D
1 µm

that each of the monoclonal antibodies recognized sp56. 2-D SDS-PAGE analysis of radiolabeled protein bound to monoclonal antibody 7C5 is presented in Fig. 4 *A*. This immunoabsorbed protein comigrated with unlabeled sp56. Each of the five monoclonal antibodies gave identical results when tested by immunoadsorption (data not shown). Western blot analysis of total, Triton-extracted proteins from Percoll-purified mouse sperm revealed that each of the monoclonal antibodies recognized denatured, nonreduced sp56 on Immobilon P membranes (Fig. 4 *B*). Each monoclonal antibody recognized both members of the sp56 doublet (best seen in Fig. 4 *B*, lanes *3* and *4*). None of the monoclonal antibodies recognized denatured, DTT-reduced sp56 on Western blots (data not shown).

Both affinity-purified and biochemically purified sp56 were subjected to Western blot analysis using monoclonal antibody 7C5 as primary antibody. Both purified proteins were recognized by the antibody (Fig. 5).

Localization of sp56 on the Mouse Sperm

Immunohistochemical studies were performed to determine the location of sp56 on mouse sperm. Silver-enhanced immunogold staining of fixed sperm (monoclonal antibody 7C5 detected by G-GAMIG) revealed that sp56 is located on the dorsal region of the sperm head (Fig. 6 *A*). Considerable range in staining intensity of silver-enhanced colloidal gold was observed. This probably represents an artifact of the silver enhancement procedure, since immunogold staining (no silver enhancement) of sp56 at the EM level revealed uniform distribution of gold particles over the dorsal region of the sperm head (see below). Both capacitated and noncapacitated sperm gave identical staining patterns. sp56 was detected by antibodies on acrosome-intact sperm (Fig. 6 *A*), but not acrosome-reacted sperm (data not shown). No staining was observed when normal mouse serum was used as primary antibody (Fig. 6 *B*). The pattern of staining observed in Fig. 6 *A* was identical for all five monoclonal antibodies, but less intense when monoclonal antibodies 4B2 and 12D2 were used (data not shown). This was likely caused by relative low affinity of these antibodies for sp56 (see Materials and Methods).

Immunogold stained sperm (monoclonal antibody 7C5 detected by LG-GAMIG) observed on EM replicas confirmed and extended light microscope observations. LG-GAMIG particles uniformly decorated the extracellular portion of the sperm head plasma membrane and were largely confined to the dorsal region overlying the acrosome (Fig. 6, *C* and *D*). Approximately 88% of particles where $n = 426$ were observed on the surface of the acrosomal segment, and no particles were observed on midpiece or tail. No gold particles were observed on sperm treated with normal mouse IgG as primary antibody (data not shown).

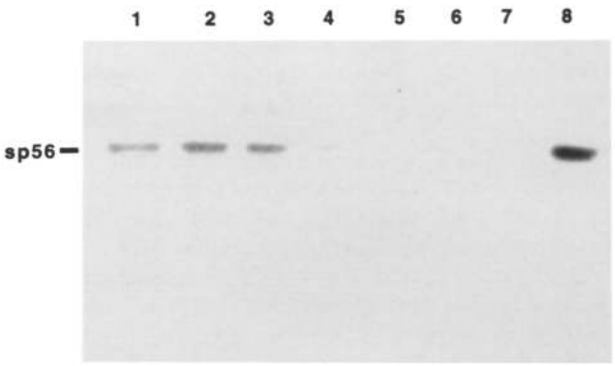


Figure 7. Differential extraction of sp56 from mouse sperm. Sperm were incubated in acidic or near-neutral buffers, as described in Materials and Methods. Buffers having pH values of 2.7 (lanes *1* and *5*), 3.0 (lanes *2* and *6*), 3.5 (lanes *3* and *7*), and 7.4 (lanes *4* and *8*) were tested for their ability to dissociate sp56 from the sperm surface. Proteins released into buffers during incubation (lanes *1–4*) and Triton-soluble proteins retained on the sperm (lanes *5–8*) were subjected to 1-D SDS-PAGE under nonreducing conditions in parallel with a lane containing reduced, prestained standards. Proteins were electroblotted onto Immobilon P membrane, the membrane was exposed to monoclonal antibody 7C5 (40 μg/ml), and the bound antibody was detected by alkaline phosphatase–conjugated goat anti–mouse IgG, as described in Materials and Methods. The position of sp56 (indicated) was identified on the basis of its migration (M_r 40,000).

Differential Extraction of Mouse Sperm

Cauda epididymal sperm released sp56 after incubation in acidic buffer (Fig. 7, lanes *1–3*). Proteins that remained associated with sperm after treatment with low pH buffers contained little sp56 (Fig. 7, lanes *5–7*), indicating that virtually the entire population of sp56 molecules was susceptible to release from sperm under acid conditions. Results shown in Fig. 7 also demonstrated that only a small amount of sp56 was released from sperm treated with buffer near neutral pH (lane *4*, released; lane *8*, retained on sperm). As described in Materials and Methods, none of the buffer treatments resulted in extensive cell lysis, indicating that release of sp56 in low pH buffer was caused solely by disruption of its contact with the sperm surface.

sp56 was also released from sperm under low salt (10 mM Tris-HCl, pH 7.4, 0–10 mM NaCl), but not under high salt (10 mM Tris-HCl, pH 7.4, 0.1–2 M NaCl) conditions (data

Figure 6. Location of sp56 on mouse sperm. (*A*) Light microscope localization of sp56 using anti-sp56 monoclonal antibody. Acrosome-intact, fixed mouse sperm were dried onto glass slides, exposed to monoclonal antibody 7C5, and bound antibody was detected by silver-enhanced G-GAMIG, as described in Materials and Methods. Black silver grains, indicating positive reaction, are located on the dorsal region of each sperm head. The slide was lightly counterstained with Coomassie blue and photographed using a green filter. (*B*) Same as *A* using normal mouse IgG as primary antibody. (*C*) EM localization of sp56, using anti-sp56 monoclonal antibody. Replicas of mouse sperm that had been bound to monoclonal antibody 7C5, followed by LG-GAMIG, were prepared and observed in the electron microscope, as described in Materials and Methods. Gold particles, seen as black dots, are largely confined to the region of the sperm head overlying the acrosome. That region is an apparent plateau or ridge on the dorsal part of the sperm head. (*D*) High magnification image of the acrosomal region seen in *C*.

not shown). However, this result may be an artifact, since low salt treatment resulted in red blood cell lysis (data not shown).

Discussion

Studies presented here were designed to further test the hypothesis (Bleil and Wassarman, 1990) that sp56 is the mouse sperm protein responsible for recognition of ZP3. If sp56 (or any other protein) is the sperm's ZP3 recognition protein, it must have at least the following characteristics: (*a*) the protein must be a sperm protein; (*b*) the protein must have specific affinity for ZP3 and ZP3's FD oligo; and (*c*) the protein must be located on the plasma membrane overlying the sperm head and colocalize with ZP3-binding sites on the sperm head.

Mouse sperm released from cauda epididymes are contaminated with varying amounts of antibody-producing cells, macrophages, red blood cells, cytoplasmic bodies, and cellular debris (unpublished observations; see Bleil and Wassarman, 1986). For this reason, initial studies designed to identify the sperm's ZP3 recognition protein and most biochemical studies presented here were performed with Percoll-purified mouse sperm (Bleil and Wassarman, 1990).

Crosslinking and affinity chromatography studies identified sp56, a mouse sperm protein located on the sperm head plasma membrane which has specific affinity for ZP3 (Bleil and Wassarman, 1990). Affinity-purified sp56, subjected to 2-D SDS-PAGE migrated as expected of a protein having intramolecular disulfide bonds (i.e., above the diagonal on such gels), consistent with an extracellular location (Fig. 1 *A*). When Triton-extracted proteins from acrosome-intact sperm were subjected to 2-D SDS-PAGE, sp56 migrated to a unique position clearly separated from other major proteins (Fig. 1 *B*). NH_2-terminal amino acid sequencing confirmed that only sp56 migrated to this position on such gels. Densitometric analysis of such gels indicated that sp56 is present at an estimated 25,000 copies per cell, consistent with the number of ZP3 binding sites on acrosome-intact sperm (Bleil and Wassarman, 1986). sp56 was undetectable on gels containing proteins extracted from acrosome-reacted sperm. The absence of sp56 on acrosome-reacted sperm is expected, since acrosome-reacted sperm are devoid of ZP3-binding sites (Bleil and Wassarman, 1983; Bleil and Wassarman, 1986; Bleil and Wassarman, 1990).

Photoactivated crosslinking of NHS-ASA-modified ZP3 glycopeptides was used to identify sperm proteins having specific affinity for ZP3's FD oligo. Percoll-purified, acrosome-intact mouse sperm were challenged with total radiolabeled glycopeptides of ZP3. Sperm selectively bound only one class of glycopeptide (~3,900-D oligosaccharide chain), which coeluted with ZP3's FD oligo (Fig. 2 *A*). This result confirmed previous studies, demonstrating selective affinity of sperm for ZP3's ~3,900-D FD oligo (Florman and Wassarman, 1985). Photoactivated cross-linking studies performed with NHS-ASA-modified total glycopeptides of ZP3 indicated that sp56 was the protein responsible for this selective affinity (Fig. 2 *B*). In addition to sp56, label was incorporated into material migrating at the stacking/separating interphase of these gels (Fig. 2 *B*, >300,000 D). Although we cannot exclude the possibility that this high molecular mass material is another protein having affinity for the FD oligo, this material most likely represents cross-linked aggregates, since cross-linking experiments using NHS-ASA commonly generate high molecular weight aggregates (unpublished observations). We believe formation of such aggregates may be caused by the fact that high frequency UV (260–300 nm) is required to activate NHS-ASA. In this connection, it should be noted that soluble ZP3 (the protein from which modified glycopeptides are derived) modified with ^{125}I-Denny-Jaffe reagent, which does not commonly form large aggregates and is activated at 360 nm, photoactivatibly cross-links to only sp56 (Bleil and Wassarman, 1990).

Relatively large amounts (micrograms) of sp56 were purified by ion exchange and size-exclusion chromatography to obtain sufficient quantities for screening and testing of anti-sp56 monoclonal antibodies (Fig. 3). The biochemically purified protein, identified on the basis of its unique migration in 2-D SDS-PAGE, was shown to be bona fide sp56: both biochemically and affinity-purified sp56 were recognized by monoclonal antibodies directed against the biochemically purified protein (Fig. 5), and both proteins had the same NH_2-terminal amino acid sequence.

A screen of the Protein Information Resources Data Base revealed the NH_2-terminal sequence of sp56 has 67% identity to the first 12 amino acids of human complement 4b-binding protein and 25% identity to mouse complement 4b-binding protein. However, the amino acid sequence derived from a tryptic peptide of sp56 showed no significant homology to either mouse or human complement 4b-binding proteins. It will be of interest to determine whether additional amino acid sequence of sp56 evidences significant homology to complement 4b-binding protein, since this protein is a member of a superfamily of protein receptors (Reid, et al., 1986).

Purified sp56 eluted from size-exclusion columns at a size class of ~110,000 D (Fig. 3 *C*). No other equally abundant protein coeluted with sp56 in such columns. Since the protein elutes as expected of a multimer in the presence of total, Triton-extracted sperm proteins (see Results), we conclude that native sp56 is a homomultimer, possibly a dimer or trimer. The observation that sp56 is a homomultimer immediately suggests a possible mechanism for ZP3-induced patching (see Introduction). Each ZP3 polypeptide chain bearing two or more FD oligos could tether at least two sp56 multimers (via contact between individual FD oligos and sp56 monomers of separate multimers). Multiple, multivalent interactions at the sperm surface would lead to aggregation (patching) of sp56, which in turn, would generate the signal to trigger membrane fusion. This possible patching mechanism is consistent with the following observations: (*a*) soluble ZP3 but not ZP3 glycopeptides induces the sperm acrosome reaction (Florman et al., 1984) and (*b*) inability of ZP3 glycopeptides to induce the acrosome reaction can be overcome by dimerizing those glycopeptides with IgG directed against ZP3 polypeptide chain (Leyton and Saling, 1989*a*). Although this proposed patching mechanism is consistent with the patching hypothesis, it should be noted that evidence for patching of soluble ZP3 (along with the sperm's ZP3 recognition protein) on the sperm head has only been inferred and has not been directly demonstrated (Wassarman et al., 1985; Leyton and Saling, 1989*a*; Bleil, 1991).

sp56 migrated as a doublet after separation by 2-D SDS-

PAGE. Both members of the sp56 doublet copurified on ZP3 affinity columns (Fig. 1) and after both ion exchange and size-exclusion column chromatography (Fig. 3). Each member of the doublet was recognized by anti-sp56 monoclonal antibodies on Western blots (Fig. 4). These results, combined with the observation that a single NH_2-terminal amino acid sequence was obtained from the sp56 doublet, suggest that both members of the doublet are closely related. The apparent size difference between the two doublet members may be caused by differences in posttranslational modification of one protein or differences in the lengths of COOH termini of two largely identical proteins. Studies are currently underway to test these possibilities.

Five monoclonal antibodies have been developed, each of which recognized sp56 (Figs. 4 and 5). All monoclonal antibodies recognized denatured, nonreduced sp56 on Western blots, but none of these antibodies recognized denatured, reduced sp56 (Fig. 4 *B*). Apparently, each of the monoclonal antibodies recognizes an sp56 epitope, the secondary or tertiary structure of which can be refolded (or is retained) after denaturation in SDS, but only when intramolecular disulfides remain intact. We have not determined whether any or all of the selected monoclonal antibodies recognize the same epitope(s) on sp56. These studies are currently in progress.

sp56 is located on the extracellular potion of the sperm head plasma membrane. Previous studies demonstrated that soluble ZP3, photoactivatably cross-linked to live, acrosome-intact sperm, was located solely on the sperm head and cross-linked solely to SP56 (Bleil and Wassarman, 1990). Additional evidence that sp56 polypeptide is exposed on the extracellular portion of plasma membrane was provided by the finding that this protein was sensitive to surface labeling methods and was removed from acrosome-intact sperm by light trypsin treatment (Bleil and Wassarman, 1990). In studies presented here, anti-sp56 monoclonal antibodies were used to confirm that sp56 epitopes are exposed on the sperm surface and to demonstrate that the protein is confined to the acrosomal region of acrosome-intact sperm (Fig. 6 *A*). sp56 was not detected on acrosome-reacted sperm. Replica EM of immunogold-stained sperm localized sp56 at higher resolution, revealing that the protein is exposed on the extracellular plasma membrane of acrosome-intact sperm and is largely confined to that region of the plasma membrane overlying the acrosome (Fig. 6, *C* and *D*).

sp56 is located on the sperm head, where ZP3 binding sites are located. Mouse sperm ZP3-binding sites bound to ZP3 colloidal gold particles are largely confined to the sperm plasma membrane surface overlying the acrosome (Mortillo and Wassarman, 1991). EM immunogold studies presented here demonstrate that sp56 is largely confined to that same region of the sperm head (Fig. 6, *C* and *D*). Current studies are designed to determine whether sp56 and the sperms' ZP3 binding sites are identical.

sp56 is a peripheral membrane protein that can be released from the sperm surface in the absence of detergents. Differential extraction of sp56 from cauda epididymal mouse sperm demonstrated that the protein was completely released from the sperm surface in low pH buffers, but was not released from sperm in near-neutral pH buffer (Fig. 7). These results indicate that sp56 is not an integral membrane protein, but is attached to the sperm surface by interactions sensitive to low pH (e.g., hydrogen bonds). It is tempting to speculate that sp56 is anchored to the plasma membrane through an integral membrane protein. If true, this model implies that the role of sp56 in sperm-egg interaction is solely to recognize and bind to ZP3. Once bound to the ZP, the hypothetical integral membrane protein might transmit the signal required to trigger membrane fusion and the acrosome reaction.

A number of other proteins have been proposed as candidates for the mouse sperm's ZP3 recognition protein. β-1,4 galactosyl transferase and a 95-kD, phosphotyrosine-bearing protein have been demonstrated to interact with mouse ZP3. Anti–cow milk galactosyl transferase antiserum binds to both plasma and inner acrosomal membranes of acrosome-intact and acrosome-reacted mouse sperm, respectively (Lopez et al., 1985), inconsistent with the absence of ZP3-binding sites on acrosome-reacted sperm (Bleil and Wassarman, 1986; Bleil et al., 1988; Mortillo and Wassarman, 1991). Cauda epididymal cells have been demonstrated to transfer ^3H from [^3H]UDP-galactose to ZP3 (Miller et al., 1992), but whether or not this activity is caused by sperm β-1,4 galactosyl transferase has not been determined. Radiolabeled, soluble ZP3 was found to bind to a denatured, reduced, blotted 95-kd protein from cauda epididymal cells (Leyton and Saling, 1989*b*). Neither β-1,4, galactosyl transferase nor the 95-kD protein has been shown to recognize ZP3's FD oligo, and neither protein has been isolated from purified mouse sperm.

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