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Identification of a ZP3-binding protein on acrosome-intact mouse sperm by photoaffinity crosslinking

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ABSTRACT **During the process of fertilization in mammals, sperm bind in a relatively species-specific manner to the zona pellucida (ZP) of ovulated eggs. ZP3, a glycoprotein found in the mouse egg zona pellucida, serves as receptor for sperm during gamete adhesion. We report here that a M_r 56,000 protein found on mouse sperm has properties expected for a sperm component that recognizes and binds to ZP3. This sperm protein is radiolabeled preferentially by a photoactivatable heterobifunctional crosslinker ("Denny–Jaffee reagent") covalently linked to purified ZP3, binds very tightly to ZP3-affinity columns, and is localized to heads of acrosome-intact but not acrosome-reacted sperm. These and other findings suggest that this protein may be a "ZP3-binding protein" that, together with the sperm receptor, supports species-specific binding of mouse sperm to unfertilized eggs.**

Mammalian eggs are encompassed by a thick extracellular coat called the zona pellucida (ZP) to which sperm bind in a relatively species-specific manner during the course of fertilization (1, 2). Sperm bind by their head to sperm receptors present in the ZP. In mice, ZP glycoprotein ZP3 ($M_r \approx 83{,}000$) serves as the primary receptor to which acrosome-intact sperm bind (by the plasma membrane overlying their heads) during gamete adhesion (3–6). Sperm bound to ZP3 then undergo the acrosome reaction, a form of exocytosis, which results in loss of plasma membrane overlying the anterior region of the sperm head (4–7). Acrosome-reacted sperm remain bound to the ZP by interacting with another ZP glycoprotein, ZP2 ($M_r \approx 120{,}000$), which serves as a secondary sperm receptor (6, 8). Bound sperm can then penetrate the ZP, probably by using a proteinase associated with the inner acrosomal membrane, and fuse with egg plasma membrane to form a zygote.

ZP3 plays at least two roles during the fertilization process in mice. It serves as primary sperm receptor and as acrosome reaction-inducer. The sperm receptor function of ZP3 apparently is solely dependent on a specific size class of serine/threonine- (O-) linked oligosaccharides ($M_r \approx 3900$) present on the glycoprotein (3–6, 9, 10). The oligosaccharides have a galactose residue at their nonreducing terminus (in α-linkage with the penultimate sugar) that is essential for sperm receptor function (10, 11). The acrosome reaction-inducer function of ZP3 is also dependent on the glycoprotein's O-linked oligosaccharides, but its polypeptide chain plays a role as well (10, 12). Therefore, the sperm plasma membrane protein that binds to ZP3 apparently does so by binding to specific carbohydrate determinants on ZP3.

Here, we describe results of experiments aimed at identifying the mouse sperm protein that recognizes and binds to ZP3 ("ZP3-binding protein") during gamete adhesion. Purified ZP3 was covalently modified with a radiolabeled, photoactivatable, heterobifunctional crosslinker ("Denny–Jaffee reagent") (13–15), incubated with acrosome-intact and acrosome-reacted sperm, and then subjected to photolysis. Electrophoretic analyses of detergent-solubilized sperm proteins that were radiolabeled after photolysis indicate that ZP3 binds preferentially to a M_r 56,000 protein present on the heads of only acrosome-intact sperm. This sperm protein has other properties as well that make it a likely candidate for the role of ZP3-binding protein. A preliminary report of these results has appeared (16).

MATERIALS AND METHODS

Modification of ZP3 with Denny–Jaffee Reagent. Mouse oocyte ZP3 (2 μg) was purified as described (8, 17) and treated with 500 μCi (1 μCi = 37 kBq) of ^{125}I-labeled Denny–Jaffee reagent, *N*-{4-(*p*-azido-*m*-[^{125}I]iodophenylazo)benzoyl}-3-aminopropyl-*N'*-oxysulfosuccinimide ester (New England Nuclear; 2000 Ci/mmol), in 50 μl of sodium bicarbonate (pH 8.3) for 1 hr at 25°C in the dark (14). The crosslinker reacts with protein amino groups during this step. Samples were brought to pH 9.0 by addition of ammonium acetate and were desalted on a Bio-Gel P-4 column (2 ml) equilibrated with buffer I [50 mM Tris chloride, pH 7.4/150 mM NaCl/1 mM $CaCl_2$/1 mM $MgCl_2$/1 mM KCl/50 μM 5-quinilinyl benzoate (QNB; Hoffmann–La Roche)/0.1 mg of bovine serum albumin per ml]. QNB is a specific inhibitor of the ZP-induced acrosome reaction (18). The void volume, containing ZP3 covalently linked to ^{125}I-labeled Denny–Jaffee reagent (^{125}I-DJ-ZP3), was stored in the dark for up to 20 min prior to addition of sperm.

Photoactivated Crosslinking of ZP3 to Sperm. Mouse sperm were obtained from cauda epididymes of two 12- to 16-week-old male mice (CD-1; Charles River Breeding Laboratories) and were capacitated in M199-M medium as described (17). Capacitated sperm were brought to 25°C and centrifuged at 1500 × *g* for 5 min; the resulting pellet was resuspended in buffer I. One hundred microliters of buffer I was added to half of the sperm suspension ("experimental" sample), and 100 μl of buffer I containing 1 μg of purified ZP3 was added to the other half ("control" sample). Both samples were mixed for 10 min and then centrifuged at 1500 × *g* for 5 min. Supernatants were discarded, and the sperm was resuspended in buffer I containing ^{125}I-DJ-ZP3. Both samples, containing $\approx 2 \times 10^7$ sperm and ≈ 1 μg of radiolabeled ZP3 in ≈ 500 μl, were mixed for 10 min in the dark. The sperm suspensions were centrifuged at 1500 × *g* for 5 min. Supernatants were photoactivated and stored for subsequent analysis, and sperm pellets were resuspended in 1 ml of buffer I and photoactivated. Photoactivation was for 2 min at 25°C with a high-pressure mercury lamp (Zeiss; 50 watt); wavelengths below 340 nm filtered out.

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Abbreviations: ZP, zona pellucida; CHAPS, 3-[3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; ^{125}I-DJ-ZP3, ZP3 derivative of I-labeled Denny–Jaffee reagent.

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Fractionation of Photoactivated Sperm. Photoactivated sperm suspensions, prepared as described above, were layered onto preformed Percoll gradients (70% Percoll and 50 mM Tris chloride, pH 7.4/150 mM NaCl/1 mM EGTA, centrifuged at 35,000 × *g* for 30 min in a Beckman 50 Ti rotor) and centrifuged at 8000 × *g* for 7 min at 25°C to separate acrosome-intact from acrosome-reacted sperm. The former banded about 50% and the latter 67% of the way down the gradient. Sperm were removed from the gradient by puncturing tubes with a syringe needle, and the purity and acrosomal status of sperm were determined as described (19). Sperm suspensions were diluted 1:4 with 50 mM Tris chloride, pH 7.4/150 mM NaCl/1 mM EGTA and then centrifuged at 3000 × *g* for 4 min. A small portion of the pellet was prepared for whole-mount autoradiography as described (17), and the remainder was used to fractionate sperm proteins crosslinked to ZP3.

Fractionation of Sperm Proteins Crosslinked to ZP3. Sperm, prepared as described above, were extracted with 100 μl of 50 mM Tris chloride, pH 7.5/150 mM NaCl/1% sodium dodecyl sulfate (SDS) at 25°C. Extracts were spun to remove insoluble material and diluted 1:9 with 50 mM Tris chloride, pH 7.5/150 mM NaCl/0.6% Nonidet P-40. Crosslinked ZP3 then was purified by immunoadsorption on rabbit anti-mouse ZP3-IgG-Sepharose. IgG was prepared from a ZP3 antiserum (8) by affinity chromatography on a ZP3 column (see below) after preadsorption with formaldehyde-fixed sperm. The purified IgG, which reacted only with ZP3 on immunoblots (Western blots) of ZP glycoproteins and did not bind to sperm as determined by silver-enhanced ImmunoGold staining, was covalently linked to Sepharose and used for immunoadsorption as described (17). Crosslinked ZP3 was eluted from IgG-Sepharose by treatment with 1% SDS at 60°C for 10 min. A portion of the ZP3, crosslinked to sperm proteins, was stored for further analysis. The remainder was treated with sodium dithionite to cleave crosslinks between ZP3 and sperm proteins. At 15-min intervals, 1.5 M sodium dithionite was added to samples to a final concentration of 0.2 M as described (14). After the final addition of sodium dithionite, samples were incubated at 25°C for 10 hr. Finally, samples were desalted on a Bio-Gel P-6 column (1 ml) in SDS–gel sample buffer [0.125 M Tris chloride, pH 6.8/2% SDS/5% (vol/vol) glycerol/50 mM dithiothreitol] and subjected to electrophoretic and autoradiographic analysis as described (17).

Affinity Chromatography of Sperm Proteins. Capacitated, acrosome-intact sperm (purified on Percoll gradients) were washed by centrifugation in 50 mM Tris chloride, pH 7.0/150 mM NaCl/1 mM $CaCl_2$/1 mM $MgCl_2$/1 mM KCl and were extracted in the same buffer containing 2% 3-[3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS) and several proteinase inhibitors (130 μM bestatin, 0.3 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin, and 1 mg of turkey egg-white trypsin inhibitor per ml) (extraction buffer). After centrifugation to remove insoluble material, sperm proteins solubilized from ≈8×10^7 sperm in 2 ml were chromatographed on a Dynamax HPLC affinity column (0.25 ml; Rainin Instruments) to which either ZP3 (100 μg) or ZP2 (170 μg) was covalently linked. After application of sperm proteins, columns were washed with ≈40 column volumes of wash buffer (extraction buffer containing 0.5% CHAPS and no proteinase inhibitors) at a flow rate of 0.2 ml/min. Bound protein was eluted with a urea gradient (0–8 M) in wash buffer.

RESULTS

Experimental Rationale. Previously we reported that purified ZP3, radioiodinated (^{125}I) to high specific activities by the chloramine-T procedure, binds specifically to heads of acrosome-intact but not acrosome-reacted sperm *in vitro* (17). Binding of ^{125}I-labeled ZP3 to acrosome-intact sperm was inhibited by preincubation of sperm in the presence of nonradioactive ZP3. In view of these findings, here we used a radiolabeled, photoactivatable, heterobifunctional crosslinker (^{125}I-labeled Denny–Jaffee reagent) (refs. 13–15; New England Nuclear) to identify the complementary sperm protein(s) to which ZP3 binds. Purified ZP3 was covalently linked to ^{125}I-labeled Denny–Jaffee reagent in the dark, converting the glycoprotein into a radiolabeled photoaffinity probe. The ZP3 derivative (^{125}I-DJ-ZP3) was then incubated with sperm in the dark and subjected to photolysis, which resulted in formation of a covalent linkage between ^{125}I-DJ-ZP3 and one or more sperm proteins. Purified ZP3-derivatized sperm were extracted with detergent, and solubilized sperm proteins linked to ZP3 were purified by immunoadsorption on ZP3-IgG-Sepharose. Purified proteins were then treated with sodium dithionite to release ZP3 (by reduction of the azo linkage) and to retain radiolabel only on sperm protein. The radiolabeled proteins were then analyzed by SDS/PAGE and autoradiography.

Whole-Mount Autoradiographic Analysis of Sperm Crosslinked to ZP3. Sperm incubated with ^{125}I-DJ-ZP3 and then subjected to photolysis to form covalent bonds between sperm protein and ZP3 were fractionated on Percoll gradients to separate acrosome-intact and acrosome-reacted sperm and

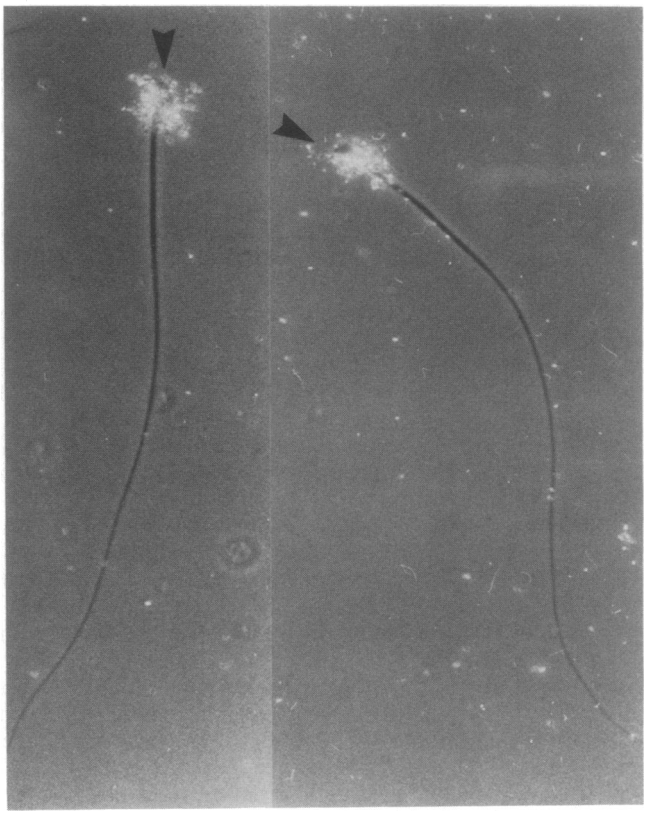

FIG. 1. Whole-mount autoradiographic visualization of ^{125}I-DJ-ZP3 covalently bound to acrosome-intact sperm. Sperm were incubated in the presence of ^{125}I-DJ-ZP3 in the dark, subjected to photolysis, and fractionated on Percoll gradients to separate acrosome-intact and acrosome-reacted sperm. Acrosome-intact and acrosome-reacted sperm were prepared for whole-mount autoradiography, and developed autoradiograms were photographed under Nomarski DIC optics as described (17). A minimum of 100 acrosome-intact and acrosome-reacted sperm were scored for the presence of silver grains overlying their heads. Shown are two typical examples of acrosome-intact sperm with silver grains overlying their heads (arrowheads). Acrosome-reacted sperm had only background levels of silver grains associated with their heads.

were subjected to whole-mount autoradiography. In these experiments, sperm were not exposed to sodium dithionite to maintain the covalent linkage between radiolabeled Denny–Jaffee reagent and ZP3. Autoradiograms revealed that acrosome-intact but not acrosome-reacted sperm had grains associated with their heads and not with the midpieces or tails (Fig. 1). These results are completely consistent with the previous finding that ^{125}I-labeled ZP3 was able to bind to heads of acrosome-intact but not acrosome-reacted sperm *in vitro* (17). Apparently, the presence of Denny–Jaffee reagent does not interfere with binding of ZP3 to heads of acrosome-intact sperm.

Electrophoretic Analysis of Sperm Proteins Crosslinked to ZP3. Identification of the sperm-head protein(s) to which ^{125}I-DJ-ZP3 was covalently bound following photoactivation was determined by SDS/PAGE and autoradiography after treatment of the samples with sodium dithionite. The latter treatment resulted in reduction of the ^{125}I-DJ-ZP3 azo linkage (14, 15) and, consequently, in transfer of radiolabel from derivatized ZP3 to sperm protein. Results of these experiments are shown in Fig. 2 and can be summarized as follows. (*i*) Purified ZP3 (molecular weight: average apparent, ≈83,000; range, <70,000 to >100,000; refs. 3–6) was heavily radiolabeled by Denny–Jaffee reagent (lane A) and, as expected for a heterogeneously glycosylated glycoprotein (6), migrated as a broad band on SDS/PAGE. (*ii*) Sodium dithionite reduction of ^{125}I-DJ-ZP3 resulted in release of virtually all radiolabel from ZP3 (lanes A and E). (*iii*) In the absence of sodium dithionite treatment, high molecular weight radiolabeled complexes were found in the stacking gel and at the origin of the separating gel following SDS/PAGE of acrosome-intact sperm to which ^{125}I-DJ-ZP3 was covalently bound after photolysis (lane B). As much as 90% of the radiolabel (estimated by microdensitometry) was associated with the stacking gel and origin of the separating gel (see *Discussion*). (*iv*) As a result of sodium dithionite reduction, several radiolabeled proteins were found in the separating gel following SDS/PAGE of acrosome-intact sperm to which ^{125}I-DJ-ZP3 had been covalently bound after photolysis (lane F). The most prominent band, representing as much as 90% of radiolabel on the gel (estimated by microdensitometry), migrated at $M_r \approx 56{,}000$; this is consistent with the results shown in lane B (see *Discussion*). It should be noted that, depending on experimental conditions (e.g., different concentrations of Denny–Jaffee reagent), the amount of radiolabel associated with the M_r 56,000 sperm protein varied between about 40% and 90% of total radiolabel incorporated into sperm protein. (*v*) Pretreatment of acrosome-intact sperm with ZP3 prevented the binding of ^{125}I-DJ-ZP3 to sperm and, consequently, the transfer of radiolabel to sperm protein after sodium dithionite treatment (lanes C and G). It should be noted that in these experiments, sperm were washed extensively to remove unbound ZP3, prior to addition of ^{125}I-DJ-ZP3. These observations are consistent with those made in analogous experiments in which sperm, pretreated with nonradioactive ZP3, were incubated with ^{125}I-labeled ZP3 and subjected to whole-mount autoradiography (17). Such pretreatment greatly reduced the amount of radiolabeled ZP3 associated with sperm heads. (*vi*) In the case of acrosome-reacted sperm, covalent binding of ^{125}I-DJ-ZP3 and transfer of radiolabel to sperm proteins were reduced by as much as 95% as compared with acrosome-intact sperm (lanes D and H). This is consistent with the previous finding that purified ZP3 binds only to heads of acrosome-intact sperm (17). Loss of plasma membrane overlying the anterior region of the sperm head, as a result of the acrosome reaction, precludes binding of ZP3. These crosslinking results (Fig. 2), taken together with the results of whole-mount autoradio-

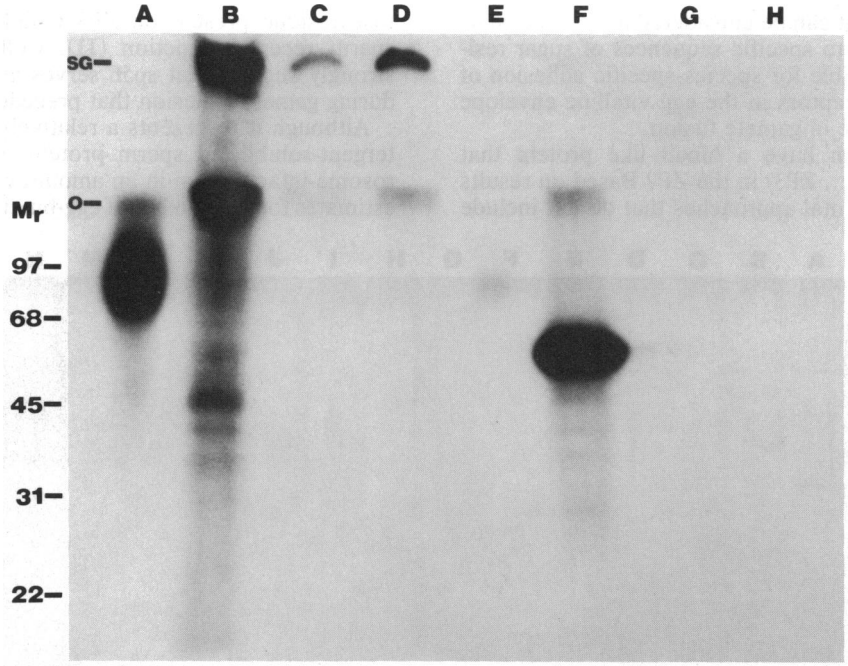

FIG. 2. Autoradiogram of electrophoretic analysis of a photoaffinity crosslinking experiment. Samples were prepared for and subjected to SDS/PAGE under reducing conditions as described. In these experiments, samples were either treated (lanes E–H) or not treated (lanes A–D) with sodium dithionite. Lanes: A and E, ^{125}I-DJ-ZP3 (≈0.5 ng) with (lane E) and without (lane A) sodium dithionite treatment; B and F, detergent-solubilized sperm proteins from acrosome-intact sperm (≈10^6 sperm) treated with ^{125}I-DJ-ZP3, immunopurified, and either treated (lane F) or not treated (lane B) with sodium dithionite; C and G, same as lanes B and F except that the acrosome-intact sperm were exposed to nonradioactive ZP3 prior to ^{125}I-DJ-ZP3, and then detergent-solubilized, immunopurified sperm proteins were either treated (lane G) or not treated (lane C) with sodium dithionite; D and H, same as lanes B and F except that acrosome-reacted sperm (≈10^6 sperm) were used in the experiment, and detergent-solubilized, immunopurified sperm proteins were either treated (lane H) or not treated (lane D) with sodium dithionite. Size markers, including phosphorylase b (M_r 97,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), and soybean trypsin inhibitor (M_r 22,000), were silver-stained following electrophoresis. sg, Stacking gel; o, origin of separating gel.

graphic analyses described above (Fig. 1), suggest that purified ZP3 associates primarily with a M_r 56,000 protein present on plasma membrane overlying heads of acrosome-intact sperm.

Affinity Chromatographic Analysis of Solubilized Sperm Proteins. The crosslinking experiments just described suggest that a M_r 56,000 sperm protein may be a ZP3-binding protein. In an alternative experimental approach, detergent-solubilized sperm proteins were subjected to ZP3-affinity chromatography to determine whether or not a M_r 56,000 protein was present that would bind tightly to ZP3. Solubilized sperm proteins bound to ZP3 were eluted by using a 0–8 M urea gradient and were detected by silver-staining following SDS/PAGE under reducing conditions. A relatively large number of sperm proteins, most with M_r values between 36,000 and 70,000, were bound to ZP3 (Fig. 3). However, the molecular weight of one of the most tightly bound proteins, which was eluted from ZP3-affinity columns with the urea gradient between 5 and 8 M, was 56,000 (Fig. 3, lanes I–O). This protein did not bind to ZP2-affinity columns (data not shown), consistent with the relatively poor binding of ZP2, as compared with ZP3, to heads of acrosome-intact sperm *in vitro* (17). Results of microdensitometry of silver- and Coomassie-stained gels suggest that the M_r 56,000 protein represents only about 0.02% and 0.004% of total CHAPS- and SDS-extractable sperm protein, respectively. Therefore, it is not an abundant sperm protein, consistent with previous estimates of the number of ZP3 binding sites (10,000–40,000) on acrosome-intact mouse sperm (17).

DISCUSSION

Bindin, a M_r 24,000 protein that coats the surface of the sea urchin sperm acrosomal process, is a well-characterized sperm receptor-binding protein (20–24). It is an extremely hydrophobic protein that can be considered a lectin because it recognizes and binds to specific sequences of sugar residues. Bindin is responsible for species-specific adhesion of sea urchin sperm to receptors in the egg vitelline envelope and may also play a role in gamete fusion.

Do mammalian sperm have a bindin-like protein that recognizes receptors (i.e., ZP3) in the ZP? Based on results of a variety of experimental approaches that do not include the use of chemical crosslinkers, several different sperm proteins have been proposed as candidates for the role of ZP3-binding protein in mammals. These proteins include galactosyl- and fucosyl-transferases (25, 26); fucose- and galactose-binding proteins (27–29); α-mannosidase (30), proacrosin, and other proteinases (31–33); and a variety of other sperm proteins identified solely on the basis of their M_r values on SDS/PAGE (34–38). In mice, the most recently proposed candidate is a M_r 95,000 sperm protein that reportedly binds specifically to ZP3 on Western blots and, in addition, is a tyrosine kinase substrate (39). Our own attempts to identify a mouse sperm protein that binds specifically to ZP3 on Western blots have proved unsuccessful, although we do not have a satisfactory explanation for this lack of success. Consequently, as described here, we turned to photoaffinity crosslinking and affinity chromatography as alternative experimental approaches by which to identify (a) ZP3-binding protein(s) on mouse sperm.

The M_r 56,000 sperm protein described here, which we call "sp56," has properties expected for a ZP3-binding protein. For example, sp56 is heavily radiolabeled by photoaffinity crosslinking of sperm with ^{125}I-DJ-ZP3 (Fig. 2), binds very tightly to ZP3-affinity columns (Fig. 3), and is present only on the heads of acrosome-intact, not acrosome-reacted, sperm (Figs. 1 and 2). The latter finding, taken together with the unlikely possibility that ^{125}I-DJ-ZP3 penetrates the sperm surface, suggests that sp56 is a plasma membrane protein. Although some other sperm proteins, including actin, can bind to purified ZP3 *in vitro* (Figs. 2 and 3; unpublished observations), only sp56 satisfies many of the requirements of a ZP3-binding protein. In addition to the observations presented here, results of preliminary experiments in our laboratory (16) suggest that sp56 binds to galactose-affinity columns but not to *N*-acetylglucosamine-affinity columns—a result consistent with the essential role of a terminal galactose residue present on ZP3 O-linked oligosaccharides in sperm receptor function (11). Collectively, these results strongly suggest that sp56 serves as a ZP3-binding protein during gamete adhesion that precedes fertilization.

Although it represents a relatively low percentage of detergent-solubilized sperm protein, sp56 is present on acrosome-intact sperm in an amount consistent with previous estimates for the number of egg-binding protein molecules on

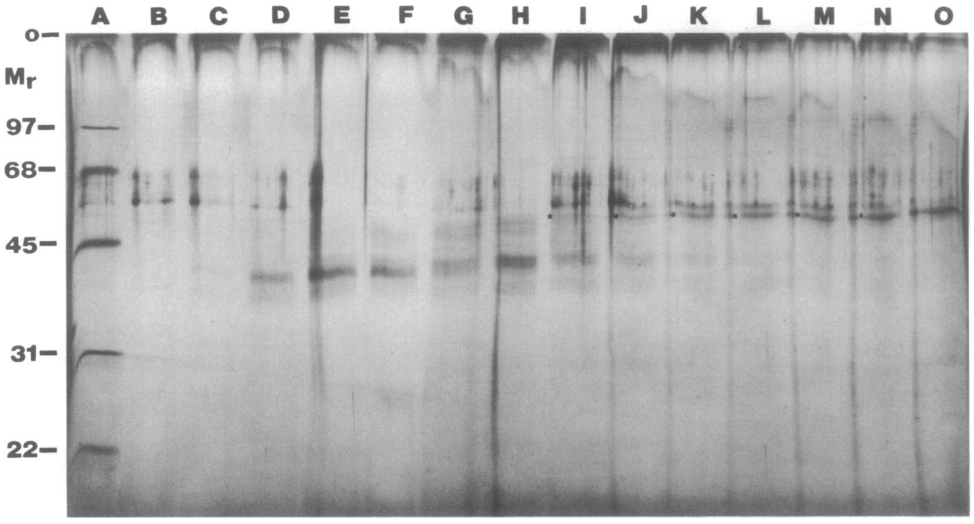

FIG. 3. Electrophoretic analysis of a ZP3-affinity chromatography experiment. Detergent-solubilized proteins from acrosome-intact sperm (≈2 × 10^7) were fractionated by ZP3-affinity chromatography with a urea gradient (0–8 M) and analyzed by SDS/PAGE as described. Shown is a silver-stained gel of such an analysis. Lanes: A, protein standards, as in the legend to Fig. 2; B, final fraction of wash prior to application of urea gradient; C–O, fractions eluted from the initial application of urea gradient (lane C) to 8 M urea (lanes N and O). The position of a M_r 56,000 protein is indicated by a black dot in lanes I–O (≈5–8 M urea). It should be noted that a band at M_r 59,000 is present in all experimental sample lanes (lanes B–O) and apparently is a contaminant, not a sperm protein.

mouse sperm (10,000–40,000 molecules) (17). In addition, although present in relatively low amounts on sperm, sp56 is by far the major radiolabeled protein observed (as much as 90% of incorporated radiolabel) after photoaffinity crosslinking with ^{125}I-DJ-ZP3 (Fig. 2). The specificity for radiolabeling by ^{125}I-DJ-ZP3 is indicated by the failure of sperm preincubated with ZP3 (in the presence of QNB, which prevents induction of the acrosome reaction) to be significantly radiolabeled by photoaffinity crosslinking (Fig. 2). In addition to sp56, a number of other sperm proteins are radiolabeled by ^{125}I-DJ-ZP3 but to a much lesser extent. This is not too surprising, since proteins in close proximity to sp56 could be nonspecifically radiolabeled by the relatively long (≈12 Å) crosslinking reagent. The possibility that more than one sperm protein supports binding of sperm to ZP3 must also be considered.

It is of interest to note that several of the previously reported candidates for ZP3-binding protein (described above; refs. 25–39) have molecular weight values similar to that of sp56. For example, boar sperm proacrosin (M_r 53,000) (31), guinea pig sperm PH-20 antigen (M_r 59,000–66,000) (37), rat sperm galactose-binding protein ("galactosyl receptor"; M_r 49,000–54,000) (29), and mouse sperm galactosyl transferase (M_r 57,000–60,000) (25) all have molecular weight values close to that of sp56. All of these proteins are reported to be located on the surface of acrosome-intact sperm heads. It remains to be determined whether or not sp56 is in fact one of the aforementioned sperm proteins.

Binding of acrosome-intact mouse sperm to ZP3 induces them to undergo the acrosome reaction (4–7), a form of exocytosis. Certain evidence suggests that induction of the acrosome reaction involves multivalent interactions between ZP3 and its binding protein, and that such interactions culminate in "capping" or "patching" of ZP3-binding proteins on sperm plasma membrane overlying the acrosome (40, 41). In view of these possibilities, we need to examine whether or not sp56 exists as a multimeric protein in sperm plasma membrane and whether or not it undergoes redistribution in the membrane during the course of the ZP3-induced acrosome reaction. Evidence from recent ultrastructural studies suggests that massive lateral displacement of transmembrane sperm proteins overlying the acrosome is, indeed, an early step in the acrosome reaction (42). In this context, it is tempting to suggest that these features of sp56 account for the high molecular weight crosslinked species that remain in the stacking gel prior to dithionite treatment (Fig. 2, lane B). Since, on average, the derivatized ZP3 molecules contained more than one crosslinker, there was an opportunity to bind more than one sp56 molecule per ZP3 molecule. If sp56 exists as a multimer in sperm plasma membrane and/or capping of sp56 occurs subsequent to binding of ZP3 to sperm, it is likely that those ZP3 molecules containing more than one crosslinker would react with more than one sp56 molecule.

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