Autoradiographic Visualization of the Mouse Egg's Sperm Receptor Bound to Sperm

Jeffrey D. Bleil and Paul M. Wassarman

Department of Cell Biology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110; and Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. The authors' present address is Roche Research Center in New Jersey.

Abstract. The extracellular coat, or zona pellucida, of mammalian eggs contains species-specific receptors to which sperm bind as a prelude to fertilization. In mice, ZP3, one of only three zona pellucida glycoproteins, serves as sperm receptor. Acrosome-intact, but not acrosome-reacted, mouse sperm recognize and interact with specific O-linked oligosaccharides of ZP3 resulting in sperm–egg binding. Binding, in turn, causes sperm to undergo the acrosome reaction; a membrane fusion event that results in loss of plasma membrane at the anterior region of the head and exposure of inner acrosomal membrane with its associated acrosomal contents. Bound, acrosome-reacted sperm are able to penetrate the zona pellucida and fuse with the egg's plasma membrane (fertilization). In the present report, we examined binding of radioiodinated, purified, egg ZP3 to both acrosome intact and acrosome reacted sperm by whole-mount autoradiography. Silver grains due to bound ^{125}I-ZP3 were found localized to the acrosomal cap region of heads of acrosome intact sperm, but not to heads of acrosome-reacted sperm. Under the same conditions, ^{125}I-fetuin bound at only background levels to heads of both acrosome-intact and -reacted sperm, and ^{125}I-ZP2, another zona pellucida glycoprotein, bound preferentially to acrosome-reacted sperm. These results provide visual evidence that ZP3 binds preferentially and specifically to heads of acrosome intact sperm; properties expected of the mouse egg's sperm receptor.

SHORTLY after mouse sperm and eggs are combined in vitro, under conditions that support fertilization, sperm attach loosely and nonspecifically to the egg's extracellular coat, or zona pellucida (Hartmann et al., 1972). Such attachment can lead to binding; a tight, relatively species-specific association between plasma membrane of the sperm head and sperm receptors of the zona pellucida (Hartmann and Hutchison, 1974; Gwatkin, 1977; Wassarman and Bleil, 1982; Bleil and Wassarman, 1983; Wassarman et al., 1985*a*, *b*). Subsequently, bound sperm undergo the acrosome reaction, whereby plasma membrane at the anterior region of the sperm head fuses with outer acrosomal membrane, exposing the inner acrosomal membrane and its associated acrosome contents (Saling et al., 1979; Saling and Storey, 1979; Florman and Storey, 1982; Bleil and Wassarman, 1983). Acrosome-reacted sperm that remain bound then penetrate the zona pellucida and reach the perivitelline space, perhaps by using the inner acrosomal membrane-associated protease, acrosin (McRorie and Williams, 1974; Green and Hockaday, 1978; Castellani-Ceresa et al., 1983). A single sperm fuses with the egg's plasma membrane (fertilization). The egg responds to fertilization by releasing cortical granule contents (cortical reaction) into the zona pellucida, altering it such that sperm are unable to bind or penetrate this extracellular coat (zona reaction) (Hartmann et al., 1972; Inoué and Wolf, 1975; Gwatkin and Williams, 1976; Schuel, 1978; Sato, 1979; Bleil and Wassarman, 1980*a*).

The mouse egg's zona pellucida is composed of only three different glycoproteins, called ZP1, ZP2, and ZP3 that are organized into long, interconnected filaments (Bleil and Wassarman, 1980*b*; Greve and Wassarman, 1985). ZP3 has been identified as the sperm receptor, primarily on the basis of an in vitro "competition assay," and as inducer of the acrosome reaction (Bleil and Wassarman, 1980*a*, 1983; Wassarman et al., 1985*a*, *b*). ZP3 (M_r 83,000) is synthesized and secreted by growing oocytes and consists of an M_r 44,000 polypeptide chain to which three or four N-linked and an undetermined number of O-linked oligosaccharides are added (Bleil and Wassarman, 1980*c*; Salzmann et al., 1983; Wassarman et al., 1985*b*). O-Linked oligosaccharides alone account for the sperm receptor activity of ZP3, whereas its acrosome reaction-inducing activity is dependent on polypeptide chain as well (Florman et al., 1984; Florman and Wassarman, 1985). Therefore, in mice, it would appear that a single glycoprotein is responsible for both binding and modification of sperm enabling them to reach and fuse with the egg's plasma membrane.

In this report, we present autoradiographic evidence sup-

porting our conclusion that ZP3 binds specifically to the head of acrosome-intact, but not acrosome-reacted sperm. Furthermore, we have found that while ZP2, another zona pellucida glycoprotein, binds poorly to acrosome intact sperm, it binds well to acrosome-reacted sperm. The implications of these and other observations are discussed in terms of the molecular basis of fertilization in mice. Preliminary accounts of some of these experiments have appeared (Bleil and Wassarman, 1985; Wassarman et al., 1985*b*).

Materials and Methods

Collection and Culture of Mouse Gametes

All gametes were obtained from Swiss, albino mice (CD-1; Charles River Breeding Laboratories, Wilmington, MA) as previously described (Bleil and Wassarman, 1983). Sperm were collected in Earle's modified medium 199 (GIBCO, Grand Island, NY) containing 25 mM Hepes, supplemented with bovine serum albumin (BSA) (4 mg/ml) and pyruvate (30 μg/ml) (pH 7.3; M199-M), and containing 4 mM EGTA. Sperm were washed once in M199-M containing EGTA (4 mM) by low-speed centrifugation. Washed sperm were resuspended in either M199-M or M199-M containing EGTA (4 mM) at a final concentration of 10^6 sperm per ml. Capacitation and culture of sperm, and isolation and culture of eggs and embryos, were carried out as previously described (Bleil and Wassarman, 1983).

Isolation of Zonae Pellucidae

In certain cases, zonae pellucidae were isolated individually from oocytes or 2-cell embryos by using mouth-operated micropipettes, as previously described (Bleil and Wassarman, 1980*b*). Alternatively, large numbers of zonae pellucidae ($5–20 \times 10^3$) were isolated by Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) gradient centrifugation of ovarian homogenates. Ovaries dissected from 20–30 mice (21 d old) were homogenized (Dounce homogenizer) on ice in 4 ml of a buffer (25 mM triethanolamine, pH 8.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) containing 1 mg DNase (Sigma Chemical Co., St. Louis, MO; Type III) and 1 mg hyaluronidase (Sigma Chemical Co.; Type IV). The homogenate was brought to 1% Nonidet P-40 and 0.1 mM phenylmethylsulfonyl fluoride and subjected to about 10 more strokes of the pestle. The homogenate was then brought to 1% deoxycholate, mixed with 9 ml of homogenization buffer containing Percoll (72%) in a seal-cap tube, and centrifuged at 25,000 rpm for 45 min at 4°C in a 50.1 type rotor (Beckman Instruments, Inc., Palo Alto, CA). Under these conditions, zonae pellucidae appeared as a narrow, opaque band at a density of ~1.02 g/ml, whereas insoluble ovarian tissue was found in the region 1.10–1.14 g/ml; densities were determined by using marker beads (Pharmacia Fine Chemicals). The band of zonae pellucidae was recovered by puncturing the side of the centrifuge tube with a syringe needle, washed twice by pelletting in 14 ml of homogenization buffer, and stored in homogenization buffer:glycerol (1:1) at −20°C at a concentration of ~75 zonae pellucidae/μl. Microscopic examination revealed that these preparations consisted of variously sized zona pellucida fragments, and electrophoretic analyses (Fig. 1) and sperm receptor activity measurements (data not shown) indicated that these preparations were virtually indistinguishable from those of individually isolated zonae pellucidae.

Radiolabeling of Zona Pellucida Glycoproteins

Intact zonae pellucidae and fetuin (Sigma Chemical Co.) were radioiodinated in phosphate buffer containing 0.1% Nonidet P-40 by the chloramine-T procedure (Amersham Corp., Arlington Heights, IL). Unincorporated ^{125}I was removed from zonae pellucidae by successive pelletting in 50 mM Tris, pH 7.4, 100 mM NaCl, and 1 mM EDTA (TNE buffer) containing 0.1% Nonidet P-40, and from fetuin by gel filtration of Bio-Gel P-6 in TNE buffer. Zona pellucida glycoproteins and fetuin were radiolabeled to about the same specific activity ($0.8–1 \times 10^8$ cpm/μg) so as to be able to compare autoradiograms directly.

Purification of Zona Pellucida Glycoproteins

Radiolabeled zonae pellucidae were solubilized in 20 μl 1% SDS for 5 min at 60°C, diluted to 250 μl with TNE buffer containing 0.6% Nonidet P-40, and cleared of insoluble material (<1% total cpm) by centrifugation. ^{125}I-ZP3 was purified by immunoadsorption to anti-ZP3-Sepharose, prepared by cross-linking CNBr-Sepharose CL-4B to a monoclonal antibody directed against ZP3 (MAb19; Greve, J. M., and P. M. Wassarman, unpublished results). 20 μl of 50% anti-ZP3-Sepharose (≈4 mg ascites fluid IgG/ml packed Sepharose), in TNE buffer containing 0.1% SDS and 0.5% Nonidet P-40, was added to the solubilized, radiolabeled zonae pellucidae and incubated for 1 h at room temperature with continuous mixing. The beads were gently pelleted (supernatant removed for purification of ZP2), washed twice in 15 ml of TNE-SDS-Nonidet-P-40 buffer, suspended in 400 μl of the same buffer, and loaded onto glass wool inside a disposable, Beckman micropipette tip. The packed beads were then washed with 200 μl of TNE and radiolabeled ZP3 eluted in 200 μl of 1% SDS. ^{125}I-ZP2 was purified from the supernatant in an identical manner by using anti-ZP2-Sepharose prepared with a monoclonal antibody directed against ZP2 (MAb35; Greve, J. M., and P. M. Wassarman, unpublished results).

Immunopurified zona pellucida glycoproteins were subjected to SDS PAGE in order to assess their purity, specific activity, and yield. Both ZP2 and ZP3 were found to be >98% pure and were not contaminated with IgG on the basis of silver-stained gels (Fig. 1). The yield of ZP2 and ZP3 ranged from 50 to 90% (five experiments) and 85 to 95% (seven experiments), respectively. Specific activities were calculated based upon the estimated amount of each glycoprotein present in zonae pellucidae and cpm present in each glycoprotein after SDS

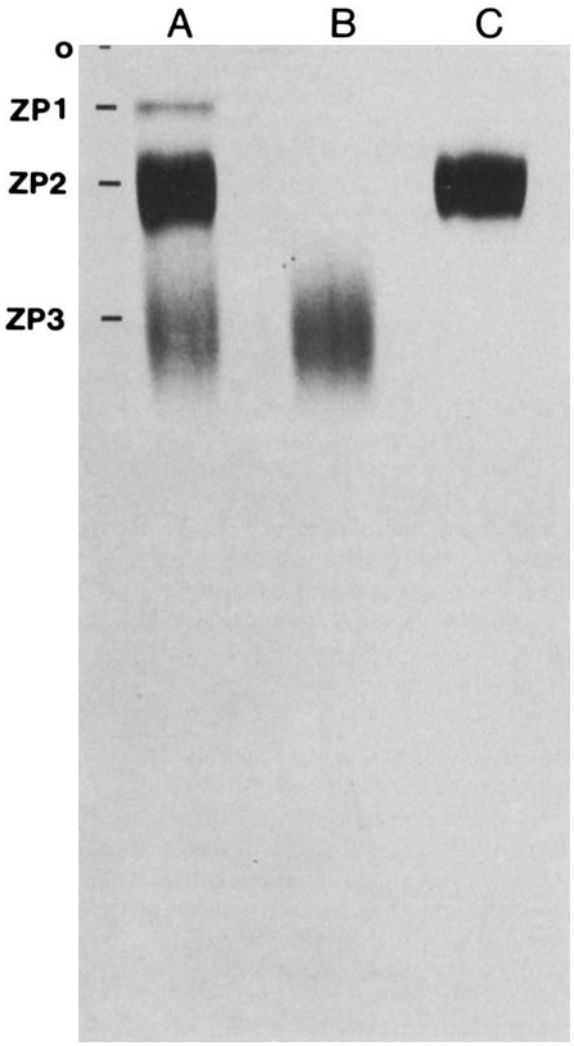

Figure 1. SDS PAGE analysis of purified zona pellucida glycoproteins. Zonae pellucidae were isolated from mouse ovaries by Percoll gradient centrifugation, and ZP2 and ZP3 purified by immunoadsorption, as described in Materials and Methods. Shown is a silver-stained gel on which isolated zonae pellucidae (*A*), immunopurified ZP3 (*B*), and immunopurified ZP2 (*C*) were run under nonreducing conditions (Bleil and Wassarman, 1980*b*). The positions of ZP1 (M_r 200,000), ZP2 (M_r 120,000), ZP3 (M_r 83,000), and the origin of the gel (*o*) are indicated.

PAGE. Purified ZP2 and ZP3 were dialyzed extensively, first against 8 M urea and then against distilled water, and lyophilized (Bleil and Wassarman, 1980*a*). Prior to use, the glycoproteins were resuspended in distilled water at 60°C for 10 min and diluted fivefold with culture medium to a final volume of ~200 μl.

Autoradiographic Analysis of Glycoprotein Binding

Capacitated sperm were added to ^{125}I-labeled proteins and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 30 min. Incubations were carried out in a final volume of 50 μl of M199-M, at a sperm concentration of 1–2 × 10^5/ml. Based simply on microscopic examination, the speed and patterns of movement of sperm used in these experiments were apparently unaffected by the radiolabeled proteins. After incubation, samples were transferred to 500 μl of Medium 199 containing 25 mM Hepes, pH 7.3, and 4 mg/ml polyvinylpyrrolidone, mixed, and 500 μl of PBS, pH 7.3, containing 2% glutaraldehyde and 2% fresh formaldehyde, was added. After gentle agitation, sperm were incubated at room temperature for 30 min, pelleted, washed four times with 50 mM triethanolamine, pH 8.0, by repeated centrifugation, and, finally, were resuspended in ~20 μl of 50 mM triethanolamine, pH 8.0. Each sample was then split into 5-μl aliquots in order to provide material for four exposure times, and each aliquot was dried onto a subbed glass slide and dehydrated through a series of ethanol washes. Approximately 25% of the original sperm (i.e., ≈300 sperm per slide) were recovered following fixation, washing, and dehydration of samples. Slides were coated with Kodak NTB-2 emulsion, diluted 1:1 with distilled water, and dried and stored for various periods of time at 4°C in the presence of Drierite. After storage, slides were developed in Kodak Dektol, mounted in PBS containing 30% glycerol and 0.1% glutaraldehyde, and observed by Nomarski differential interference contrast optics (Carl Zeiss, Inc., Thornwood, NY) in order to score each sperm for the presence or absence of an acrosome and to determine the location and number of grains associated with each sperm. Each slide contained three samples, corresponding to sperm incubated with radiolabeled ZP3, ZP2, or fetuin.

Results

Experimental Rationale

In mice, sperm first make contact with the egg itself at the surface of the zona pellucida. This contact can lead to binding of acrosome-intact sperm to eggs via sperm receptors present in zonae pellucidae and (a) component(s) of plasma membrane overlying the anterior region of the sperm head. Bound sperm then undergo the acrosome reaction, which enables them to both penetrate the zona pellucida and fuse with the egg's plasma membrane (fertilization) (see introduction; Wassarman, 1983).

Sperm exposed to purified egg ZP3 are prevented from binding to zonae pellucidae of unfertilized eggs (Bleil and Wassarman, 1980*a*; Florman et al., 1984; Florman and Wassarman, 1985*a*, *b*). Presumably, ZP3 occupies binding sites associated with plasma membrane overlying the sperm head, thereby preventing sperm from binding to zonae pellucidae. Here, we describe experiments designed to evaluate directly whether ZP3 binds to the head of acrosome-intact, but not acrosome-reacted sperm. ZP3, as well as ZP2 and fetuin, were radiolabeled with ^{125}I to high specific activities (0.8–1 × 10^8 cpm/μg) and then incubated with capacitated, motile sperm under conditions shown to support fertilization in vitro. Sperm were then processed for and subjected to whole mount autoradiography. Each sperm was scored for the presence or absence of an intact acrosome, and the location and number of grains associated with the sperm were determined.

Evaluation of Acrosome-intact and Acrosome-reacted Sperm

Two different methods were used to differentiate acrosome intact from acrosome-reacted sperm. The first involved an immunological assay identical to that described by Florman et al. (1984), except that a peroxidase conjugate of rabbit anti-mouse IgG was used as the second antibody (Jonak, 1980). The second method simply involved examination of fixed sperm by Nomarski differential interference contrast (DIC)[1] microscopy.

As previously described (Florman et al., 1984), acrosome-intact sperm treated with supernatant from hybridoma HS19A9 display regionalized binding of antibody over the acrosomal cap, whereas acrosome-reacted sperm bind undetectable levels of the monoclonal antibody (Fig. 2). Examination of >200 HS19A9-treated, acrosome-intact sperm by Nomarski DIC microscopy, revealed in every case a very prominent ridge overlying the acrosomal cap; in no case was the ridge seen with acrosome-reacted sperm (i.e., HS19A9 negative) as prominent. The difference in appearance of the ridge associated with acrosome-intact as compared to acrosome-reacted sperm was also observed by Nomarski DIC microscopy with unstained preparations. Measurements of the thickness of the ridge at its widest point indicate that the ridge associated with acrosome-intact sperm is ~2.5 times thicker than that of acrosome-reacted sperm (1.2 vs. 0.5 μm, respectively; see footnote to Table I). As seen in Fig. 2, sperm prevented from undergoing the acrosome reaction by incubation in the presence of EGTA display a much more prominent ridge than those induced to undergo the acrosome reaction by treatment with ionophore A23187. These data, some of which are summarized in Table I, demonstrate that Nomarski DIC microscopy can be used reliably to score for the presence or absence of intact acrosomes on mouse sperm.

Autoradiographic Evidence for Binding of ZP3 to Acrosome-intact Sperm

ZP3 was radiolabeled and incubated with sperm, in the presence of BSA, in preparation for whole mount autoradiography, as described in Materials and Methods. The presence of BSA eliminated virtually all nonspecific binding of glycoproteins to sperm. In these experiments, ^{125}I-labeled fetuin was chosen as a control glycoprotein since, like ZP3, it has a relatively low pI and possesses both N- and O-linked oligosaccharides (Kornfeld and Kornfeld, 1980). At the end of the incubation period, sperm were diluted, fixed, washed, and prepared for autoradiography. Individual sperm were scored for the presence or absence of an acrosome and the number of grains over the head was determined, as seen in Fig. 3. Sperm were not scored if the acrosomal region of the head was obscured by grains or the orientation of the head did not permit a side view. Time of exposure of the autoradiograms was varied and a single time, corresponding to a maximum of 55 grains per head, was chosen for analysis. Samples having >55 grains per head led to unreliable grain counts and made it difficult to identify the status of the acrosome. Approximately 150 sperm were scored within each experimental group.

Under the conditions used, fetuin did not bind to an appreciable extent to any region of sperm (Fig. 4). On the other hand, as seen in Fig. 4, grains representing bound ^{125}I-ZP3 were found associated with sperm and exclusively with the sperm head, usually anterior to the postacrosomal region. Although both red blood cells and residual bodies are present

[1] *Abbreviation used in this paper:* DIC, differential interference contrast.

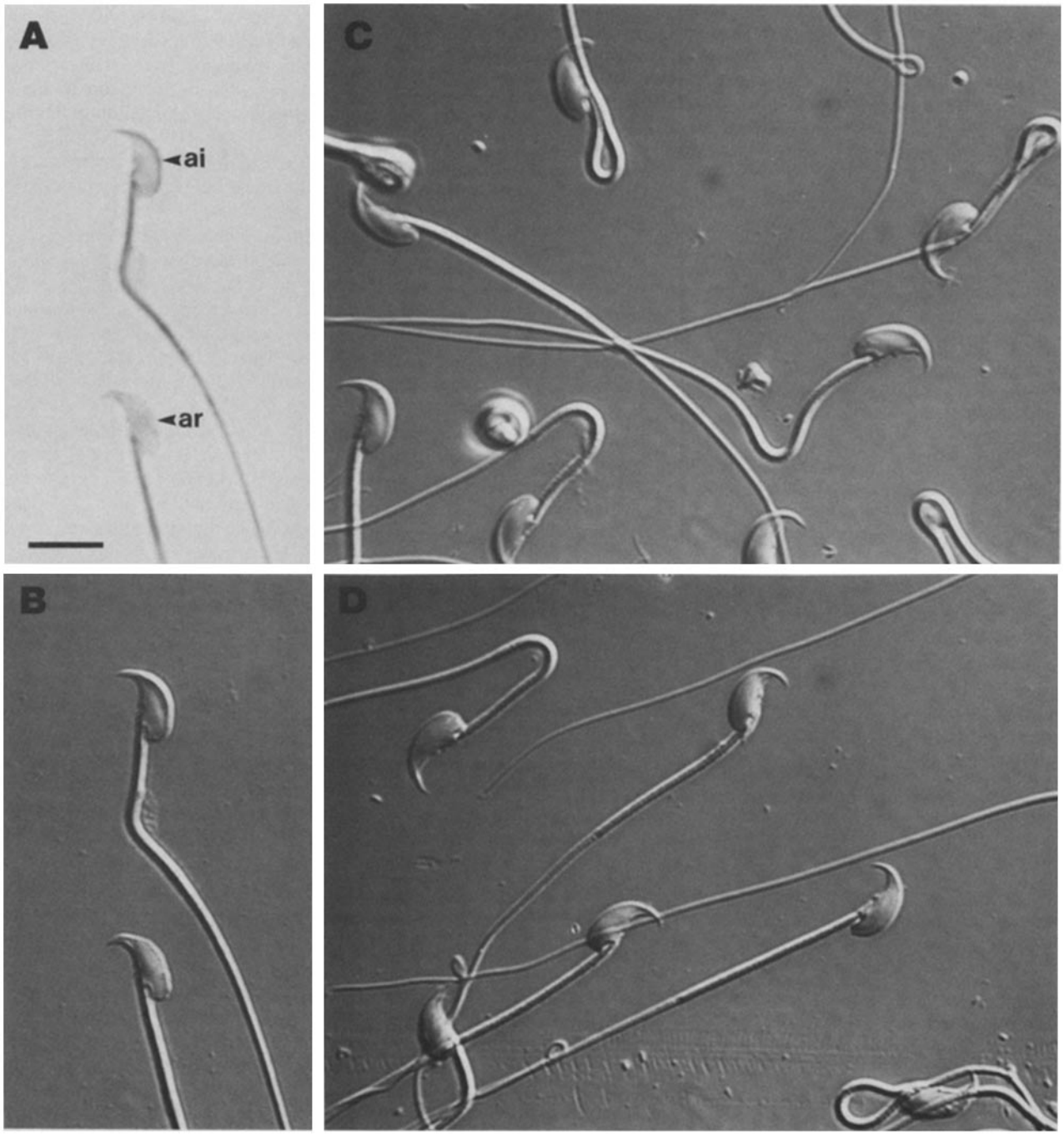

Figure 2. Identification of acrosome-intact and -reacted sperm by Nomarski DIC microscopy. Sperm obtained from a single male mouse were cultured 1 h in M199-M containing either 4 mM EGTA (*C*) or 20 μM ionophore A23187 (*A*, *B*, and *D*), and then fixed, washed, and dried on glass slides, as described in Materials and Methods. One sample of sperm was assayed for the presence or absence of intact acrosomes by the indirect immunoperoxidase method (*A* and *B*). The bright field image shown in *A* illustrates the staining of an acrosome-intact (*ai*) sperm, as compared to the lack of staining of an acrosome-reacted (*ar*) sperm. By Nomarski DIC microscopy (*B*), the stained, acrosome-intact sperm exhibits a prominent ridge overlying the acrosomal cap region of the head, whereas, the acrosome-reacted sperm does not. A second sample of the sperm preparation was examined by Nomarski DIC microscopy without immunoperoxidase staining (*C* and *D*). Sperm prevented from undergoing the acrosome reaction by treatment with EGTA (*C*) exhibited a prominent ridge overlying the acrosomal cap region of the head; whereas, those induced to undergo the acrosome reaction by treatment with ionophore A23187 (*D*) lacked a prominent ridge. These data are quantitated in Table I. Photographs were taken using a Zeiss 63× Planapo objective. Bar, 10 μm.

in the sperm preparations, no grains were found associated with these contaminants (Fig. 4). Therefore, while ZP3 and fetuin share some common molecular features, only ZP3 binds to sperm, and the binding is specific with respect to both cell type and cellular localization. It should be noted that sperm incubated in the presence of ^{125}I-ZP3, under conditions used for autoradiographic analyses, were also washed and subjected to SDS PAGE to ensure that radiolabel remained associated with ZP3. A single radioactive band, migrating as authentic ZP3 (M_r 83,000) was always observed.

Since grains representing bound ^{125}I-ZP3 were found associated with sperm, the sperm were scored for the presence or absence of intact acrosomes. To carry out these measurements, each sperm was examined by Nomarski DIC microscopy, first with a 63× Planapo objective to assess the status of acrosomes, and then, after depolarization of incident light, with a 40× Plan-Neofluar objective to visualize and count grains. In preparations containing both acrosome-intact and -reacted sperm, grains due to bound ^{125}I-ZP3 were only found associated with acrosome-intact sperm (Fig. 5) and the number of grains associated with acrosome-intact sperm increased with increasing amounts of ^{125}I-ZP3 present in the incubation mixture (Fig. 6). Quantitation of these data confirmed that binding of ^{125}I-ZP3 to acrosome-reacted sperm (~20% of the population) occurred only at background levels; i.e., ~0.2 grains per head after an 18-h exposure, at all ZP3 concentrations tested (Figs. 5 and 6). Therefore, in addition to the specificity of binding described above, ZP3 binds only to sperm with intact acrosomes.

Table I. Detection of Acrosome-reacted Sperm by Nomarski Differential Interference Contrast Microscopy

Sample	Treatment‡	No. acrosome-reacted sperm/No. total sperm (%)*	
		Immunoperoxidase	Nomarski DIC
I	EGTA	14/114 (12)	14/114 (12)
	A23187	74/97 (76)	74/97 (76)
II	EGTA	—	12/125 (10)
	A23187	—	105/144 (73)

* Sperm were scored as acrosome reacted when they did not demonstrably concentrate stain over the acrosomal cap region of the head in the immunoperoxidase assay and/or did not exhibit a prominent ridge when observed by Nomarski DIC microscopy. Measurements indicated that the average thickness of the ridge of acrosome-intact sperm, at its widest point, was 1.23 μm (n = 34; SD = 0.14 μm); whereas, the ridge of acrosome-reacted sperm was 0.52 μm (n = 31; SD = 0.10 μm) at its widest point.

‡ Sperm were exposed to either 20 μM ionophore A23187 or 4 mM EGTA, as described in the legend to Fig. 2 and in Materials and Methods.

Finally, binding of ^{125}I-ZP3 to acrosome intact sperm was examined in the presence of increasing concentrations of unlabeled, solubilized, egg zonae pellucidae (ZP1, ZP2, and ZP3). A fixed amount of radiolabeled ZP3 (10^6 cpm; 0.25 zona pellucida equivalents per μl) was incubated with sperm in the presence of various amounts of solubilized zonae pellucidae (0–1 zona pellucida per μl) and binding of ^{125}I-ZP3 to sperm evaluated by whole mount autoradiography, as above. As seen in Fig. 7, binding of ^{125}I-ZP3 to sperm was inhibited by increasing amounts of solubilized zonae pellucidae, suggesting that radiolabeled and unlabeled ZP3 compete for a finite number of binding sites on the sperm head.

Autoradiographic Evidence for Binding of ZP2 to Acrosome-reacted Sperm

We have also examined the binding of ZP2, another zona pellucida glycoprotein, to sperm, in the manner described above. Results of previous experiments strongly suggest that intact ZP2, ZP2 glycopeptides, or oligosaccharides derived from ZP2 possess neither sperm receptor nor acrosome reaction–inducing activities (Bleil and Wassarman, 1980*a*; Florman et al., 1984; Florman and Wassarman, 1985).

Autoradiography revealed that binding of ^{125}I-ZP2 to acrosome-intact sperm occurred at a relatively low level as compared to ZP3 (15–24%), but at a significantly higher level

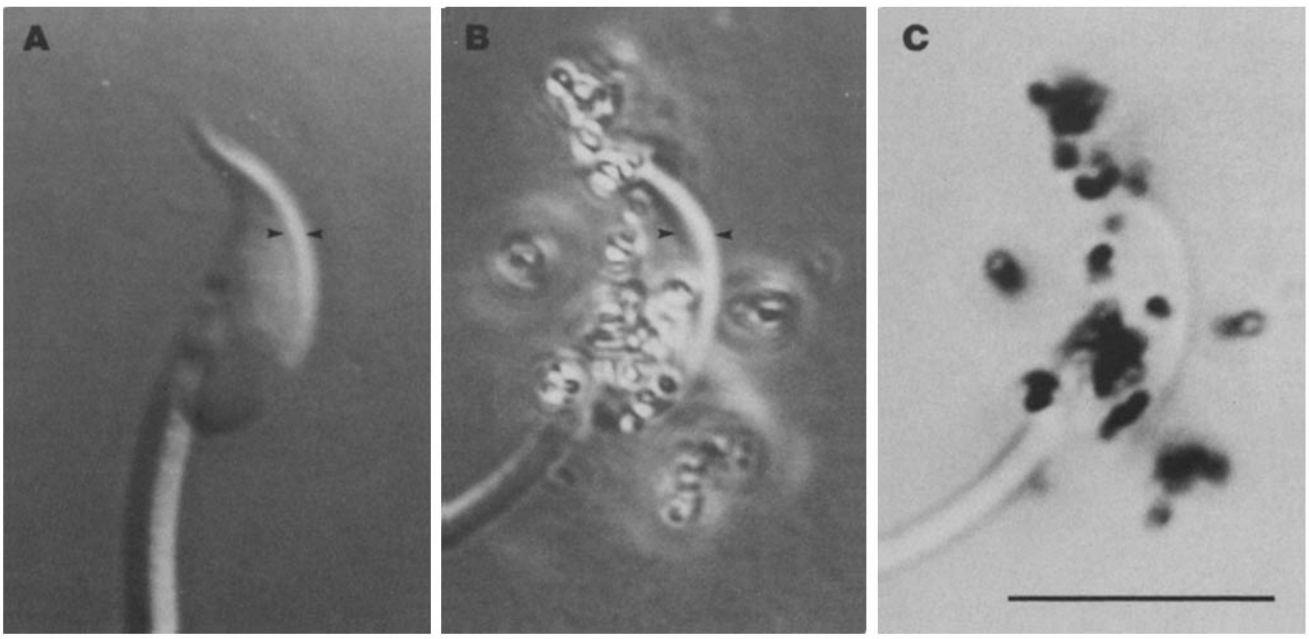

Figure 3. Identification of acrosome-intact and -reacted sperm following whole mount autoradiography. Sperm incubated in the presence of purified, ^{125}I-ZP3 (4.5 μCi/ml; 8 × 10^7 cpm/μg) in a 50-μl volume, were subjected to whole mount autoradiography, as described in Materials and Methods. Autoradiograms were exposed for 36 h at 4°C in the dark, developed, and photographed under Normarski DIC optics with a Zeiss 63× Planapo objective. Shown are an acrosome-reacted sperm (*A*) and an acrosome-intact sperm (*B* and *C*). Note that the ridge associated with the acrosomal region of the sperm head (indicated by arrowheads) in *A* is significantly narrower than that associated with the acrosomal region of the sperm head (indicated by arrowheads) in *B* (see footnote to Table I). To count the number of silver grains associated with sperm heads, the light was depolarized, as in (*C*). Shown in *C* is one of four planes of focus through the autoradiographic emulsion; a total of 29 grains were counted over this sperm head. No grains were present over the sperm head shown in *A*. Bar, 10 μm.

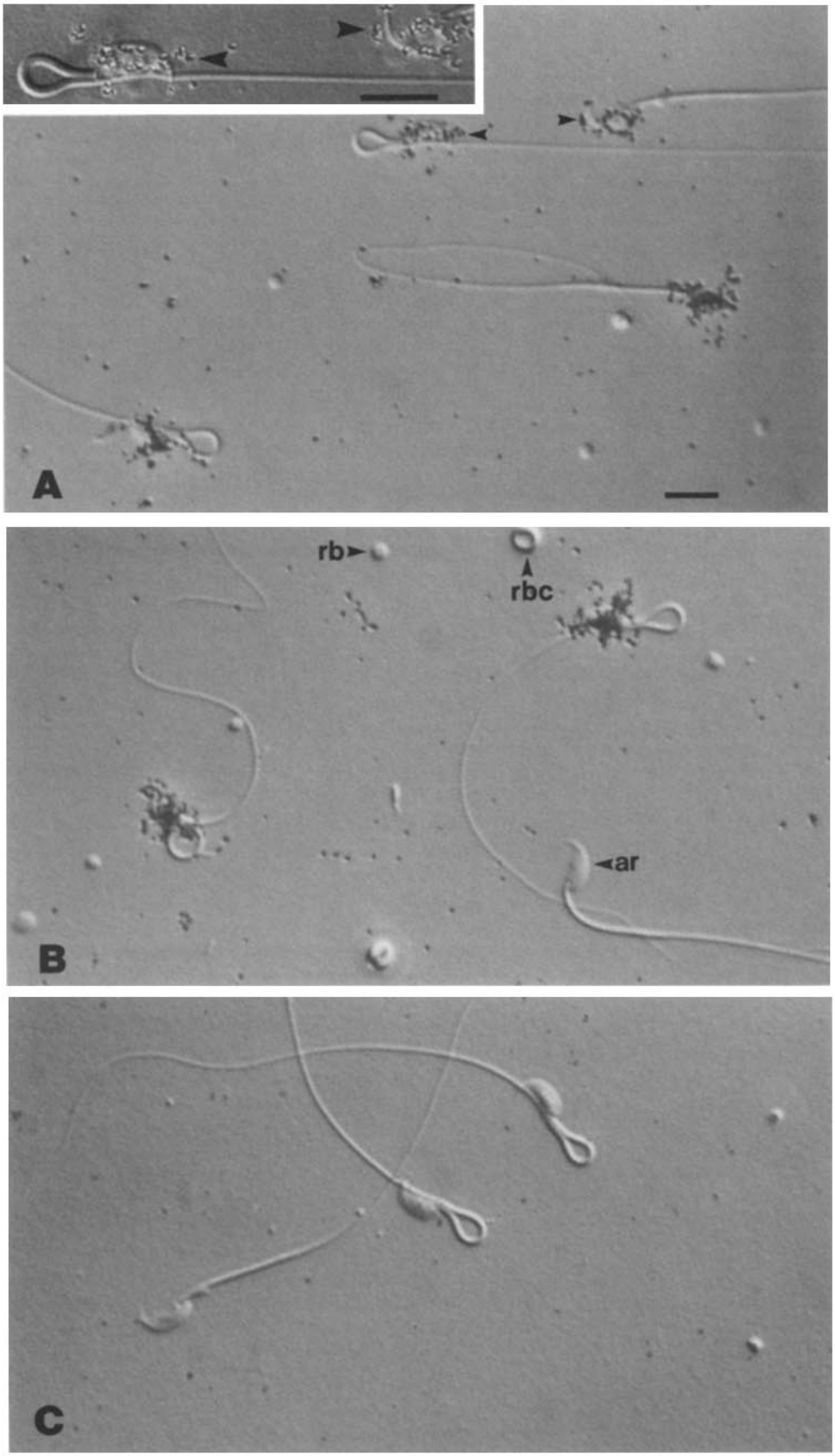

Figure 4. Autoradiographic visualization of radiolabeled sperm receptor bound to acrosome-intact sperm. Sperm incubated in the presence of either purified ^{125}I-ZP3 (4.5 μCi/ml; 8×10^7 cpm/μg (*A* and *B*) or ^{125}I-fetuin (4.5 μCi/ml; 8×10^7 cpm/μg) (*C*), in a 50-μl volume, were subjected to whole mount autoradiography, as described in Materials and Methods. Autoradiograms were exposed for 72 h at 4°C in the dark, developed, and photographed under Nomarski DIC optics with either a Zeiss 63× Planapo objective (inset, *A*) or a Zeiss 40× Plan-Neofluar objective (*A*, *B*, and *C*). Shown are representative results from a single experiment. Using standard Nomarski DIC optics (inset, *A*), sperm having as many as 50 grains localized to the head could be scored as either acrosome intact or reacted. Depolarization of the incident light permitted clear identification of grains, but was accompanied by a decrease in resolution (*A*, *B*, and *C*). *ar*, acrosome-reacted sperm; rbc, red blood cell; *rb*, residual body. Bar, 10 μm.

than the binding of ^{125}I-fetuin (Fig. 8). On the other hand, radiolabeled ZP2 bound extremely well to acrosome-reacted sperm, reaching as much as 50 times the level of binding of ^{125}I-ZP3 or ^{125}I-fetuin to the heads of acrosome-reacted sperm (Fig. 8). Grains due to bound ^{125}I-ZP2 were found over the entire sperm head anterior to the postacrosomal region. These

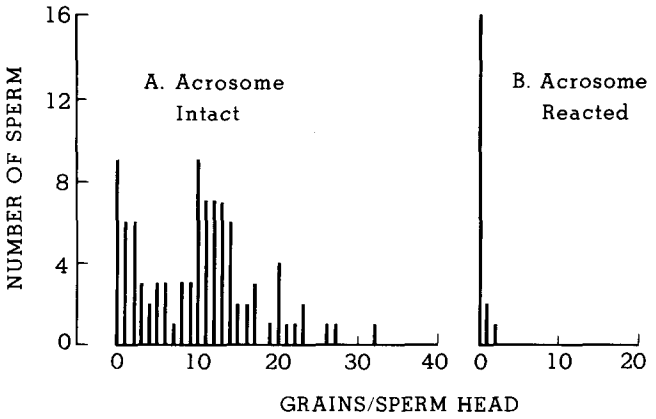

Figure 5. Quantification of autoradiographic analysis of binding of radiolabeled sperm receptor to acrosome-intact and -reacted sperm. Sperm were incubated in the presence of purified ^{125}I-ZP3 and subjected to whole mount autoradiography, as described in the legend to Fig. 4 and in Materials and Methods. Autoradiograms were exposed for 18 h at 4°C in the dark. Individual sperm were scored as acrosome intact or reacted and the number of grains associated with the head was determined. The number of sperm having a given number of grains per head is plotted for both acrosome-intact (*A*) and acrosome-reacted (*B*) sperm. In *A*, 94 sperm were scored, with an average of 10 grains per head. In *B*, 19 sperm were scored, with an average of 0.2 grains per head. These data are taken from the experiment shown in Fig. 3 and are representative of all other experiments performed.

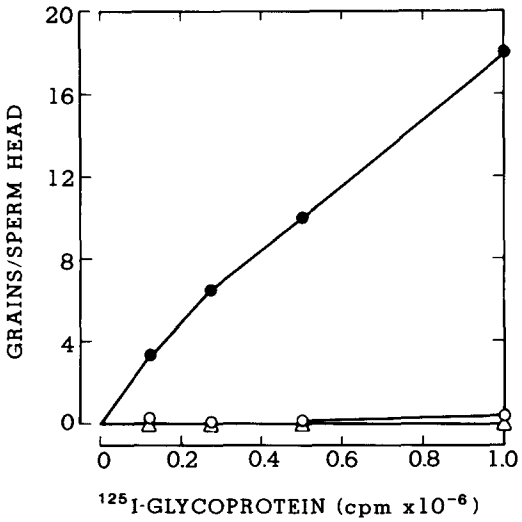

Figure 6. Binding of radiolabeled sperm receptor to acrosome-intact and -reacted sperm. Sperm were incubated in the presence of increasing concentrations of either purified ^{125}I-ZP3 (8×10^7 cpm/μg) or ^{125}I-fetuin (8×10^7 cpm/μg), in a 50-μl volume, as described in the legend to Fig. 4 and in Materials and Methods. Autoradiograms were exposed for 18 h at 4°C in the dark. Shown are the average number of grains per sperm head, plotted as a function of either ^{125}I-ZP3 (●, ○) or ^{125}I-fetuin (△) concentration, for both acrosome-intact (●, △) and -reacted (○, △) sperm.

observations strongly suggest that ZP2 binds extremely well to the inner acrosomal membrane, and/or material associated with the inner acrosomal membrane.

Discussion

We (Bleil and Wassarman, 1980*a*) and others (Gwatkin and Williams, 1976) found that pre-treatment of sperm with solubilized egg zonae pellucidae prevented binding of these sperm to unfertilized eggs, under conditions that supported fertilization in vitro. Furthermore, results of analogous experiments, using individual, purified, egg zona pellucida glycoproteins, revealed that only ZP3 prevented binding of sperm to unfertilized eggs; neither ZP1 nor ZP2 had any effect on binding (Bleil and Wassarman, 1980*a*). Comparisons of results obtained with purified ZP3 and solubilized zona pellu-

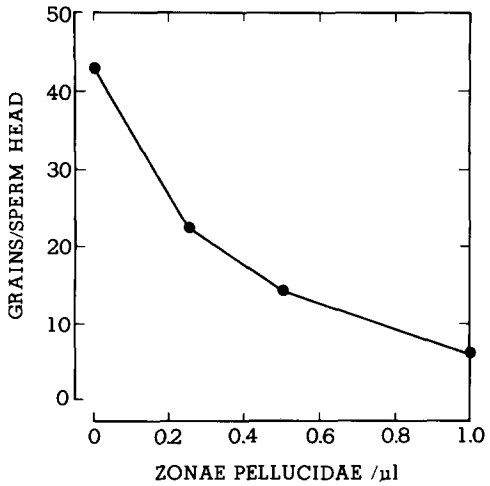

Figure 7. Inhibition of binding of radiolabeled sperm receptor to sperm by solubilized zona pellucida glycoproteins. Sperm were incubated in the presence of a constant amount of ^{125}I-ZP3 (1×10^6 cpm; 0.25 zonae pellucidae per μl) and increasing concentrations of unlabeled, solubilized zonae pellucidae (prepared as described in the legend to Fig. 1 and in Materials and Methods). Autoradiograms were exposed for 36 h at 4°C in the dark. Shown are the average number of grains per sperm head plotted as a function of solubilized zona pellucida concentration.

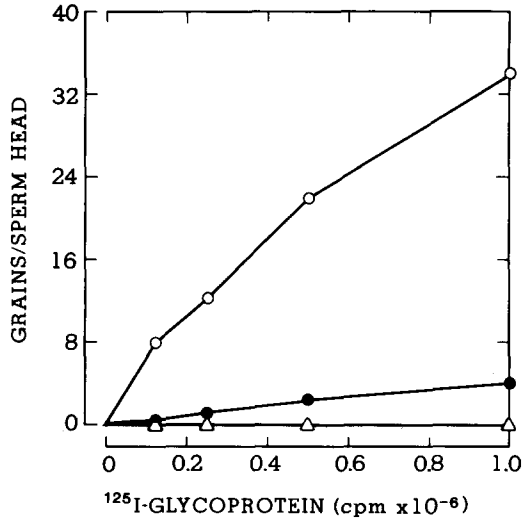

Figure 8. Binding of radiolabeled ZP2 to acrosome-intact and -reacted sperm. Sperm were incubated in the presence of increasing concentrations of either purified ^{125}I-ZP2 (1×10^8 cpm/μg) or ^{125}I-fetuin (8×10^7 cpm/μg), in a 50-μl volume, as described in the legend to Fig. 4 and in Materials and Methods. Autoradiograms were exposed for 18 h at 4°C in the dark. Shown are the average number of grains per sperm head, plotted as a function of either ^{125}I-ZP2 (●, ○) or ^{125}I-fetuin (△) concentration, for both acrosome-intact (●, △) and -reacted (○, △) sperm.

cida preparations indicated that ZP3 alone accounted for all sperm receptor activity present in egg zonae pellucidae. Overall, these observations suggest that ZP3 is the sperm receptor and, as such, can interact with sperm in vitro and prevent them from binding to the zona pellucida of unfertilized eggs. Since only acrosome-intact sperm bind to eggs (Saling et al., 1979; Saling and Storey, 1974; Florman and Storey, 1982; Bleil and Wassarman, 1983), it follows that the inhibitory effect of ZP3 is attributable to interaction with plasma membrane overlying the heads of acrosome-intact sperm.

The observations just described allow several predictions to be made about the outcome of experiments that use whole mount autoradiography to examine binding of radiolabeled ZP3 to sperm. For example, it is to be expected that ZP3 would bind specifically to the sperm head, and only to the heads of acrosome-intact sperm. Furthermore, assuming the presence of a finite number of receptor binding sites on each sperm head, unlabeled ZP3 should compete with radiolabeled ZP3 for such sites. These predictions have been borne out by the results of experiments described here.

By using autoradiographic emulsion to detect binding of ^{125}I-ZP3, we have found that ZP3 binds only to acrosome-intact, but not to acrosome-reacted sperm and binding is localized to the sperm head (Figs. 4 and 5). Under the same in vitro conditions, ^{125}I-fetuin bound to sperm at only background levels. The extent of binding of ^{125}I-ZP3 to sperm was concentration dependent (Fig. 6) and unlabeled, zona pellucida glycoproteins competed with radiolabeled ZP3 for sperm binding sites (Fig. 7). These results suggest that sperm contain a finite number of binding sites for ZP3 and that these sites are localized to plasma membrane of the sperm head. Once the acrosome reaction has taken place, resulting in loss of plasma membrane at the anterior region of the sperm head, localized binding of ZP3 is no longer observed (Figs. 4–6).

Exposure of sperm to increasing concentrations of ^{125}I-ZP3, over the range 0 to 0.26 zona pellucida equivalents per μl, resulted in increasing numbers of grains associated with sperm heads (Fig. 6). These relatively low concentrations of ZP3, as well as the short incubation time (30 min), were chosen in order to minimize induction of the acrosome reaction by the glycoprotein. In other experiments, in which ZP3 concentrations were increased to 2 zona pellucida equivalents per μl, we observed a leveling off of the number of grains per sperm head at relatively high zona pellucida concentrations (data not shown). Quantitation of these data suggest that saturation of ZP3 binding sites occurred in the range 10–50 $\times$ 10^3 ZP3 molecules bound per sperm. However, these values are somewhat suspect, in view of the possibility that a portion of the sperm population was induced to undergo the acrosome reaction at these zona pellucida concentrations (Bleil and Wassarman, 1983).

While ^{125}I-ZP3 bound to acrosome-reacted sperm at only background levels (Fig. 6), ^{125}I-ZP2 bound to these sperm extremely well (Fig. 8). This observation suggests that, as a consequence of the acrosome reaction, sites are exposed on the sperm's inner acrosomal membrane (with its associated acrosomal contents) to which ZP2 bind. It should be noted that this could account for the low, but significant level of binding of ^{125}I-ZP2 to acrosome-intact sperm (Fig. 8). Since the methods used here to score acrosomes are probably unable to detect partially reacted acrosomes, the low level of binding of radiolabeled ZP2 may actually reflect binding to inner acrosomal membrane of sperm heads in the process of undergoing the acrosome reaction. In fact, we did observe sperm heads with acrosomal ridges that appeared punctate, as if in the process of undergoing the acrosome reaction; in these cases, the sperm were scored as acrosome intact. It remains possible, however, that ^{125}I-ZP2 has specific, albeit low affinity for the plasma membrane of acrosome-intact sperm.

Sperm that have bound to the zona pellucida and, subsequently, undergone the acrosome reaction, remain bound (Bleil and Wassarman, 1983). This is in contrast to the behavior of free-swimming, acrosome-reacted sperm, which are unable to bind to zonae pellucidae (Saling et al., 1979). Results presented here suggest the possibility that ZP2 acts as a secondary receptor for sperm, interacting with inner acrosomal membrane of bound sperm following the ZP3–induced acrosome reaction. Such a situation apparently applies during fertilization in sea urchins, where a jelly coat component ("primary receptor") induces sperm to undergo the acrosome reaction and a vitelline envelope component ("secondary receptor") mediates binding of the sperm's acrosomal filament via the inner acrosomal membrane-associated protein (lectin), bindin (Vacquier and Moy, 1977; SeGall and Lennarz, 1979). If, indeed, ZP2 serves such a "secondary receptor" function, the question arises as to why free-swimming, acrosome-reacted sperm are unable to bind to zonae pellucidae. At present, the answer is not clear. However, it is tempting to speculate that the strength of the interaction between ZP2 and acrosome-reacted sperm is insufficient to bind free-swimming sperm, but sufficient to maintain binding of sperm that are already tightly bound to the surface of zonae pellucidae. Since bound, acrosome-reacted sperm must penetrate the zona pellucida in order to reach and fuse with the egg's plasma membrane, it seems reasonable that relatively weak interactions between acrosome-reacted sperm and zona pellucida would be advantageous for the sperm's progress through the extracellular coat. This and other possibilities should be considered and tested experimentally.

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References

Bleil, J. D., and P. M. Wassarman. 1980*a*. Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell.* 20:873–881.

Bleil, J. D., and P. M. Wassarman. 1980*b*. Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Dev. Biol.* 76:185–203.

Bleil, J. D., and P. M. Wassarman. 1980*c*. Synthesis of zona pellucida proteins by denuded and follicle-enclosed mouse oocytes during culture *in vitro*. *Proc. Natl. Acad. Sci. USA.* 77:1029–1033.

Bleil, J. D., and P. M. Wassarman. 1983. Sperm–egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev. Biol.* 95:317–324.

Bleil, J. D., and P. M. Wassarman. 1985. Autoradiographic visualization of localized binding of the mouse egg's sperm receptor to sperm. *J. Cell Biol.*

101(5, Pt. 2):263a. (Abstr.)

Castellani-Ceresa, L., B. Berruti, and R. Colombo. 1983. Immunocytochemical localization of acrosin in boar spermatozoa. *J. Exp. Zool.* 227:297–304.

Florman, H. M., and B. T. Storey. 1982. Mouse gamete interactions: the zona pellucida is the site of the acrosome reaction leading to fertilization *in vitro*. *Dev. Biol.* 91:121–130.

Florman, H. M., K. B. Bechtol, and P. M. Wassarman. 1984. Enzymatic dissection of the functions of the mouse egg's receptor for sperm. *Dev. Biol.* 106:243–255.

Florman, H. M., and P. M. Wassarman. 1985. O-Linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell.* 41:313–324.

Green, D. P. L., and A. R. Hockaday. 1978. The histochemical localization of acrosin in guinea pig sperm after the acrosome reaction. *J. Cell. Sci.* 32:177–184.

Greve, J. M., and P. M. Wassarman. 1985. Mouse egg extracellular coat is a matrix of interconnected filaments possessing a structural repeat. *J. Mol. Biol.* 181:253–264.

Gwatkin, R. B. L. 1977. Fertilization. *In* The Cell Surface in Embryogenesis and Development. G. Poste and B. L. Nicolson, editors. North Holland Press, Amsterdam. 1–55.

Gwatkin, R. B. L., and D. T. Williams. 1976. Receptor activity of the solubilized hamster and mouse zona pellucida before and after the zona reaction. *J. Reprod. Fertil.* 49:55–59.

Hartmann, J. F., and C. F. Hutchison. 1974. Nature of the pre-penetration contact interactions between hamster gametes *in vitro*. *J. Reprod. Fertil.* 36:49–57.

Hartmann, J. F., R. B. L. Gwatkin, and C. F. Hutchison. 1972. Early contact interactions between mammalian gametes *in vitro:* evidence that the vitellus influences adherence between sperm and zona pellucida. *Proc. Natl. Acad. Sci. USA.* 69:2767–2769.

Inoué, M., and D. P. Wolf. 1975. Fertilization associated changes in the murine zona pellucida: a time sequence study. *Biol. Reprod.* 113:546–551.

Jonak, Z. I. 1980. Peroxidase-conjugated antiglobulin method for visual detection of cell surface antigens. *In* Monoclonal Antibodies. Hybridomas: A New Dimension in Biological Analysis. R. H. Kennett, T. J. McKearn, and K. B. Bechtol, editors. Plenum Publishing Corp., New York. 378–380.

Kornfeld, R., and S. Kornfeld. 1980. Structure of glycoproteins and their oligosaccharide units. *In* The Biochemistry of Glycoproteins and Proteoglycans. W. J. Lennarz, editor. Plenum Publishing Corp., New York. 1–34.

McRorie, R. A., and W. L. Williams. 1974. Biochemistry of mammalian fertilization. *Annu. Rev. Biochem.* 43:777–803.

Saling, P. M., J. Sowinski, and B. T. Storey. 1979. An ultrastructural study of epididymal mouse spermatozoa binding to zona pellucida *in vitro*: sequential relationship to the acrosome reaction. *J. Exp. Zool.* 209:229–238.

Saling, P. M., and B. T. Storey. 1979. Mouse gamete interactions during fertilization in vitro. Chlortetracycline as a fluorescent probe for the mouse sperm acrosome reaction. *J. Cell Biol.* 83:544–545.

Salzmann, G. S., J. M. Greve, R. J. Roller, and P. M. Wassarman. 1983. Biosynthesis of the sperm receptor during oogenesis in the mouse. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:1451–1456.

Sato, K. 1979. Polyspermy preventing mechanisms in mouse eggs fertilized in vitro. *J. Exp. Zool.* 210:350–353.

Schuel, E. 1978. Secretory functions of the egg cortical granules in fertilization and development: a critical review. *Gamete Res.* 1:299–381.

SeGall, G. K., and W. J. Lennarz. 1979. Chemical characterization of the component of the jelly coat from sea urchin eggs responsible for induction of the acrosome reaction. *Dev. Biol.* 71:33–48.

Vacquier, V. D., and G. Moy. 1977. Isolation of bindin: the protein responsible for adhesion of sperm to sea urchin eggs. *Proc. Natl. Acad. Sci. USA.* 74:2456–2460.

Wassarman, P. M., and J. D. Bleil. 1982. The role of the zona pellucida glycoproteins as regulators of sperm–egg interaction in the mouse. *In* Cellular Recognition. W. A. Frazier, L. Glaser, and D. I. Gottlieb, editors. Alan R. Liss, Inc., New York. 845–863.

Wassarman, P. M. 1983. Fertilization. *In* Cell Interactions and Development: Molecular Mechanisms. K. Yamada, editor. John Wiley & Sons, Inc., New York. 1–27.

Wassarman, P. M., H. M. Florman, and J. M. Greve. 1985*a*. Receptor-mediated sperm–egg interactions in mammals. *In* Biology of Fertilization, Vol. 2. C. B. Metz and A. Monroy, editors. Academic Press, Inc., New York. 341–360.

Wassarman, P. M., J. D. Bleil, H. M. Florman, J. M. Greve, R. J. Roller, G. S. Salzmann, and F. G. Samuels. 1985*b*. The mouse egg's receptor for sperm: what is it and how does it work? *Cold Spring Harbor Symp. Quant. Biol.* 50:11–20.