Association of egg zona pellucida glycoprotein mZP3 with sperm protein sp56 during fertilization in mice

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ABSTRACT **Purified mouse sperm receptor, a zona pellucida glycoprotein called mZP3, binds to plasma membrane overlying acrosome-intact sperm heads (P.M. Wassarman, 1999, *Cell* 96, 175-183). Some evidence suggests that mZP3 binds to sp56, a protein reported to be associated peripherally with the plasma membrane of acrosome-intact sperm heads (J.D. Bleil and P.M. Wassarman, 1990, *Proc. Natl. Acad. Sci., USA* 87, 7215-7219; A. Cheng *et al.,* 1994, *J. Cell Biol.* 125, 867-878). Here, we report that membrane vesicles prepared from acrosome-intact sperm contain sp56. When these vesicles are incubated with eggs they inhibit binding of sperm to eggs *in vitro* (ID$_{50}$ ~50-100 μg protein/ml). On the other hand, a monoclonal antibody directed against sp56 relieves the inhibition of binding of sperm to eggs by membrane vesicles. As expected, incubation of intact sperm with the antibody directed against sp56 inhibits binding of the sperm to eggs. Results of immunoprecipitation of sperm extracts incubated with mZP3, by either a polyclonal antibody directed against mZP3 or a monoclonal antibody directed against sp56, suggest that mZP3 is specifically associated with sp56. Results of laser scanning confocal microscopy of fixed sperm probed with antibodies directed against either sp56 or a ~155 kDa acrosomal protein, suggest that the two proteins are present in the acrosome, but with different distributions. Furthermore, confocal images of sperm, fixed after exposure to purified mZP3 and probed with antibodies against mZP3 and sp56, reveal overlap between mZP3 and sp56 at the surface of the sperm head. The possible implications of these results are discussed in the context of mammalian fertilization.**

KEY WORDS: *mouse, eggs, sperm, zona pellucida, sp56, sperm-binding, fertilization.*

Preface

It is a great pleasure to contribute an article to a volume honoring Professor Anne McLaren for her outstanding research career. Her many experimental and intellectual contributions to reproductive and developmental biology certainly warrant such recognition. We offer our hearty congratulations and wish her many more years of enjoyment and productivity in research.

Introduction

During fertilization, species-specific binding of sperm to eggs depends upon interactions between gamete surface (glyco)proteins (Wassarman, 1987; Yanagimachi, 1994; Snell and White, 1996; Wassarman, 1999; Wassarman *et al.,* 2001). All mammalian eggs are surrounded by a thick extracellular coat, the zona pellucida (ZP), that contains species-specific receptors for sperm ("sperm receptors"). In mice, a ZP glycoprotein called mZP3 has been identified as the receptor to which acrosome-intact sperm bind (Bleil and Wassarman, 1980; Wassarman, 1990, 1999; Wassarman *et al.,* 2001). Apparently, sperm recognize and bind to specific mZP3 serine- (O-) linked oligosaccharides located close to the polypeptide's carboxy-terminus (Florman and Wassarman, 1985; Wassarman, 1990; Wassarman *et al.,* 1999; Chen *et al.,* 1998). Following binding to mZP3, sperm undergo the acrosome reaction, a form of cellular exocytosis that enables sperm to penetrate the ZP and fuse with egg plasma membrane (Wassarman, 1987; Yanagimachi, 1994; Snell and White, 1996; Wassarman, 1999;

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Wassarman *et al.,* 2001). Following gamete fusion, sperm receptors are inactivated and the ZP is hardened as part of the, so-called, zona reaction ("slow block to polyspermy").

Although the nature of the protein ("egg-binding protein", EBP) associated with sperm plasma membrane that binds to mZP3 oligosaccharides remains unresolved, many candidate EBPs have been proposed (Snell and White, 1996; Wassarman, 1999; Wassarman *et al.,* 2001). Among these is sp56, a mouse sperm protein first identified as an EBP by affinity-crosslinking (Bleil and Wassarman, 1990) and later characterized as having specific affinity for mZP3 oligosaccharides involved in sperm binding (Cheng *et al.,* 1994). sp56 has been cloned and shown on the basis of primary structure to belong to a superfamily of protein receptors possessing specific structural domains, but does not have an obvious carbohydrate recognition domain (CRD) (Bookbinder *et al.,* 1995). Despite these intriguing characteristics, evidence that sp56 may be an orthologue of an acrosomal matrix protein (called AM67) localized to the sperm acrosomal vesicle and not on the surface of the sperm head (Foster *et al.,* 1997; Hardy and Mobbs, 1999; Kim, K-S. *et al.,* 2001), has cast considerable doubt on its proposed role as an EBP.

Here, several kinds of experiments have been carried out with mouse sperm membrane vesicles and intact sperm in order to pursue potential relationships between mZP3, sp56, and binding of sperm to eggs. The results demonstrate that a monoclonal antibody directed against sp56 (anti-sp56) can interfere with binding of either sperm or sperm membrane vesicles to the egg ZP. Additionally, association of mZP3 with sp56 is indicated by results of immunoprecipitation experiments and laser scanning confocal microscopy (LSCM) using a polyclonal antibody directed against mZP3

(anti-mZP3) and anti-sp56. While it is clear that anti-sp56 recognizes antigen inside the sperm acrosomal vesicle, as reported previously (Foster *et al.,* 1997; Hardy and Mobbs, 1999; Kim, K-S. *et al.,* 2001), it would appear that sp56 also is located at sites on the surface of the sperm head where it is accessible to mZP3.

Results

Characterization of sperm membrane vesicles

Mouse sperm membrane vesicles were prepared by adopting the vortex-method of Kalab *et al.* (1994), essentially as described in Materials and Methods. From approximately 20 male mice, 2-4 months-of-age, $\sim 3 \times 10^8$ sperm were obtained which yielded the equivalent of ~150 μg of membrane vesicle protein. We determined that this procedure did not significantly affect the acrosomal status of sperm populations; for example, ~29% and ~32% of sperm were acrosome-reacted before and after the vortex procedure, respectively (determined by PSA staining, as described in Materials and Methods). Apparently, the vesicles were derived primarily from sperm plasma membrane, not acrosomal membrane. Electron micrographs of sperm membrane vesicle preparations revealed that they consisted primarily of vesicles of various sizes (Fig. 1A). Furthermore, Western immunoblots of vesicle preparations, using a monoclonal antibody directed against sperm protein sp56 (anti-sp56), revealed that the vesicles contained sp56 (Fig. 1B). Under reducing conditions, sp56 migrates with an apparent M_r of ~56 kDa on SDS-PAGE (Bleil and Wassarman, 1990), but under non-reducing conditions has an apparent M_r of ~42 kDa (Cheng *et al.,* 1994; Fig. 1B). Interestingly, membrane vesicles prepared from acrosome-reacted sperm (induced by 5 μM ionophore A23187) by the vortex-method contained less than one-tenth of the sp56 associated with vesicles from acrosome-intact sperm (Fig. 1B). Furthermore, sp56 was primarily associated with sperm heads, not with sperm tails when enriched preparations (see Materials and Methods) were examined (Fig. 1C). The characteristics of these sperm membrane vesicles permitted an analysis of the potential relationship between mZP3, sp56, and sperm binding to the ovulated egg ZP.

Inhibition of sperm binding to eggs by sperm membrane vesicles

Mouse sperm incubated in the presence of nanomolar concentrations of purified mZP3 are prevented from binding to the ZP of ovulated eggs *in vitro* (Bleil and Wassarman, 1980; Florman and Wassarman, 1985; Litscher and Wassarman, 1996). To assess whether sperm membrane vesicles can inhibit binding of sperm to eggs *in vitro,* ovulated eggs were incubated in the presence or absence of vesicles and then free-swimming, capacitated sperm were added, essentially as described in Materials and Methods. As seen in Fig. 2A, binding of sperm to eggs is inhibited by
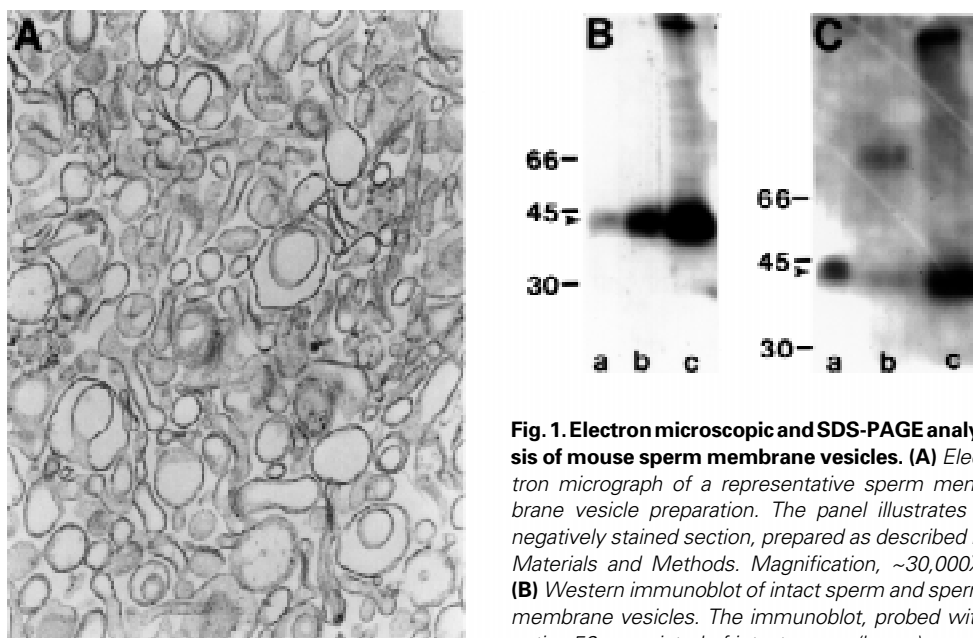

Fig. 1. Electron microscopic and SDS-PAGE analysis of mouse sperm membrane vesicles. (A) *Electron micrograph of a representative sperm membrane vesicle preparation. The panel illustrates a negatively stained section, prepared as described in Materials and Methods. Magnification, ~30,000X.* **(B)** *Western immunoblot of intact sperm and sperm membrane vesicles. The immunoblot, probed with anti-sp56 , consisted of intact sperm (lane c), membrane vesicles from acrosome-intact sperm (lane b), and membrane vesicles from acrosome-reacted sperm (lane a), prepared as described in Materials and Methods. Equivalent amounts of protein were loaded into each lane of a non-reducing SDS-PAGE gel. The position of sp56, with an apparent M_r of ~42 kDa, is indicated by an arrowhead.* **(C)** *Western immunoblot of sperm heads and tails. The immunoblot, probed with anti-sp56, consisted of intact sperm (lane c), sperm heads (lane a), and sperm tails (lane b), prepared as described in Materials and Methods. Equivalent amounts of protein were loaded into each lane of a non-reducing SDS-PAGE gel. The position of sp56 with an apparent M_r of ~42 kDa, is indicated by an arrowhead. Numbers to the left of the blots indicate size markers in kDa.*
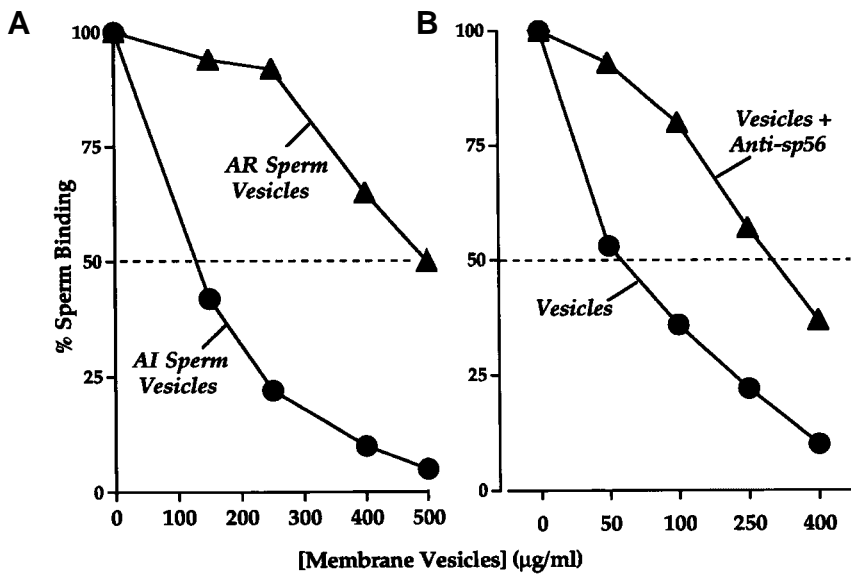
Fig. 2. Inhibition of binding of sperm to eggs by sperm membrane vesicles either untreated or treated with anti-sp56. (A) *Membrane vesicles were prepared from either acrosome-intact (AI; closed circles) or acrosome-reacted (AR; closed triangles) sperm, as described in Materials and Methods. Ovulated eggs were exposed to various amounts of membrane vesicles (150-500 μg vesicle protein/ml), capacitated sperm were added, and binding of sperm to eggs was assessed. The values reported for percent sperm binding represent the average of at least three independent experiments.* **(B)** *Membrane vesicles were prepared from acrosome-intact sperm and were used either untreated (closed circles) or treated with 10 μg/ml anti-sp56 (closed triangles), as described in panel A. In these experiments 50-400 μg of vesicle protein/ml were used. The values reported for percent sperm binding represent the average of at least three independent experiments.*

as much as 90% when eggs are first incubated in the presence of increasing amounts of membrane vesicles derived from acrosome-intact sperm (~75-80% acrosome-intact; ID_{50} ~100 μg/ml vesicle protein). On the other hand, membrane vesicles derived from acrosome-reacted sperm (~80-90% acrosome-reacted; induced by ionophore A23187) are an effective inhibitor only at significantly higher protein concentrations (ID_{50} ~500 μg/ml vesicle protein) (Fig. 2A). These results suggest that relatively low concentrations of membrane vesicles from acrosome-intact sperm bind to the egg ZP and prevent binding of sperm. Although membrane vesicles from acrosome-reacted sperm also inhibit sperm binding, at least 5-fold higher protein concentrations are required.

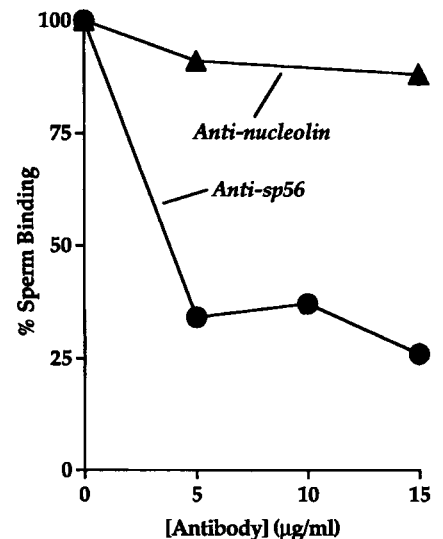### Relief of inhibition of sperm binding by anti-sp56

To determine whether sp56 plays a role in binding of sperm membrane vesicles to the egg ZP, anti-sp56 was employed. First, anti-sp56 was tested directly to determine whether it could inhibit binding of sperm to eggs *in vitro*. Capacitated sperm were incubated alone ("control") or in the presence of either anti-sp56 or an antibody directed against mouse nucleolin (anti-nucleolin) and were then exposed to ovulated eggs. While anti-nucleolin had no significant effect on sperm binding, anti-sp56 reduced binding of sperm to eggs by as much as ~70-80%, as compared to controls (Fig. 3). Consistent with these results, incubation of sperm membrane vesicles in the presence of anti-sp56 significantly reduced their ability to prevent binding of sperm to eggs (vesicles were washed extensively to remove unbound antibody prior to use in sperm-binding assays; Fig. 2B). In these experiments, the ID_{50} for vesicles alone was ~50 μg/ml, whereas the ID_{50} for vesicles incubated in the presence of anti-sp56 was ~325 μg/ml, a difference of 6- to 7-fold. It was noted in these and other experiments, that different sperm membrane vesicle preparations possessed somewhat different abilities to inhibit binding of sperm to eggs (e.g., compare ID_{50} in Fig. 1A [~100 μg/ml] with ID_{50} in Fig. 1B [~50 μg/ml]). This could reflect differences in amounts of sp56 associated with individual sperm membrane vesicle preparations. Overall, these results suggest that binding of anti-sp56 either to intact sperm or to sperm membrane vesicles prevents them from binding to ovulated eggs.
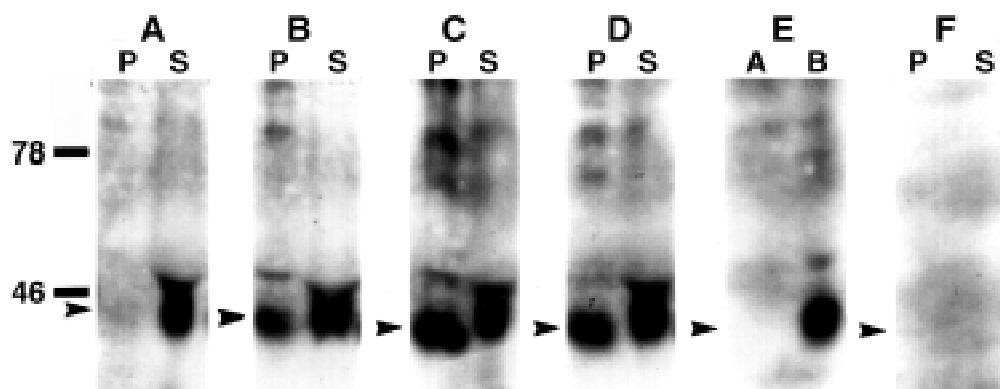
Co-immunoprecipitation of sp56 and mZP3

Previously, it was reported that sp56 is preferentially radiolabeled by a photoactivatable crosslinker (^{125}I-Denny-Jaffe reagent) covalently-linked to purified mZP3 (Bleil and Wassarman, 1990) and that sp56 binds tightly to mZP3 affinity-columns and to mZP3 oligosaccharides (Cheng *et al.,* 1994). Here, to determine whether sp56 associates with mZP3 when sperm membrane vesicles bind to eggs, immunoprecipitation experiments were carried out with anti-mZP3 and anti-sp56. Anti-mZP3 does not immunoprecipitate sp56 from sperm protein extracts and anti-sp56 does not immunoprecipitate purified mZP3 (Fig. 4). In these experiments, sperm extracts were incubated in the presence of purified mZP3, immunoprecipitated with either anti-mZP3 or anti-sp56, and precipitates and supernatants were subjected to non-reducing SDS-PAGE and Western immunoblotting (see Materials and Methods). Antibody directed against mZP3 immunoprecipitated an ~83 kDa M_r protein corresponding to mZP3 (data not shown), as well as a ~42 kDa M_r protein corresponding to sp56 under non-reducing conditions (Fig. 4, panel B; note the presence of sp56 in the precipitate [P] in panel B, but not in the precipitate [P] in panel A). Similarly, an antibody directed

Fig. 3. Effect of anti-sp56 and anti-nucleolin on binding of sperm to eggs. *Capacitated sperm were treated with different concentrations (5-15 μg/ml) of either anti-sp56 (filled circles) or anti-nucleolin (filled triangles), incubated with ovulated eggs and two-cell embryos, and the number of sperm bound to eggs determined, as described in Materials and Methods. The values reported for percent sperm binding represent the average of at least three independent experiments.*

Fig. 4. Co-immunoprecipitation of sp56 and mZP3 from mouse sperm extracts. *Capacitated sperm extracts were incubated in the presence or absence of mZP3 and then immunoprecipitated by either anti-sp56 or anti-mZP3 and analyzed by SDS-PAGE (non-reducing) and Western immunoblotting (probed with anti-sp56), as described in Materials and Methods. In each case (A-D and F), P represents the immunoprecipitate and S represents the supernatant.* **(A)** *Sperm extract incubated with anti-mZP3.* **(B)** *Sperm extract incubated with mZP3 followed by anti-mZP3.* **(C)** *Sperm extract incubated with anti-sp56.* **(D)** *Sperm extract incubated with mZP3 followed by anti-sp56.* **(E)** *Lane A contains anti-sp56 alone. Lane B contains total sperm protein alone.* **(F)** *mZP3 incubated with anti-sp56. The position of sp56 with an apparent M$_r$ of ~42 kDa, is indicated by an arrowhead.*

against sp56 immunoprecipitated a ~42 kDa M$_r$ protein corresponding to sp56, as well as an ~83 kDa M$_r$ protein corresponding to mZP3 (data not shown). As in previous reports (Bleil and Wassarman, 1990; Cheng *et al.,* 1994), these results suggest that mZP3 associates specifically with sp56 in sperm protein extracts.

Immunolocalization of sp56 and mZP3 by LSCM

While certain experiments suggest that sp56 is peripherally associated with plasma membrane overlying the heads of acrosome-intact sperm (Bleil and Wassarman, 1990; Cheng *et al.,* 1994), others suggest that sp56 is localized to the contents of the acrosomal vesicle (Foster *et al.,* 1997; Hardy and Mobbs, 1999; Kim, K-S. *et al.,* 2001). The latter condition would seem to preclude sp56 functioning as an EBP. Here, LSCM of antibody-stained, fixed mouse sperm has been used to re-examine the location of sp56. In these experiments, anti-sp56, anti-mZP3, and MC101, a monoclonal antibody directed against a mouse sperm ~155 kDa acrosomal protein (Toshimori *et al.,* 1995), have been employed (see Materials and Methods).

LSCM of fixed sperm exposed to antibody MC101 reveals that the antigen is distributed throughout the length and width of the acrosomal vesicle (Fig. 5). On the other hand, the staining pattern of fixed sperm exposed to anti-sp56 differs somewhat in that the length, but not the width of the acrosomal vesicle is stained for sp56 (Fig. 5). The narrower staining pattern for anti-sp56 suggests that the antigen is localized to only a portion of the acrosomal vesicle and to the outer acrosomal membrane, and may be present on the plasma membrane. In this context, it is of interest to note that, consistent with previous observations (Foster *et al.,* 1997; Hardy and Mobbs, 1999), we were unable to detect sp56 by LSCM with anti-sp56 on unfixed, live sperm, even after long incubation times under several conditions (data not shown).

To determine whether there is any association between mZP3 and sp56 on fixed sperm, double-labeling experiments were carried out by LSCM using FITC- (anti-rabbit) and Texas Red- (anti-mouse) conjugated secondary antibodies, as described in Materials and Methods. The staining pattern of sperm incubated with purified mZP3, fixed and then incubated with anti-mZP3 alone (data not shown) resembled that pattern reported previously for sperm incubated with gold-labeled mZP3 (Mortillo and Wassarman, 1991). However, when the sperm were probed with both anti-mZP3 (green; FITC) and anti-sp56 (red; Texas Red) the staining pattern was more complex (Figs. 6 and 7). Although sp56 was found

primarily in the acrosomal vesicle as before (Fig. 5), significant overlap of mZP3 and sp56 (yellow; FITC plus Texas Red) was observed on the surface of the sperm head (Figs. 6 and 7). This overlap was not observed when sperm were stained either for anti-mZP3 (green; FITC) and MC101 (red; Texas Red) or for anti-sp56 (red; Texas Red) and anti-mZP2 (green; FITC) directed against another ZP glycoprotein, mZP2, added to sperm (data not shown). It should be noted that mZP2 binds preferentially to acrosome-reacted sperm, not to acrosome-intact sperm (Bleil and Wassarman, 1986; Bleil *et al.,* 1988; Mortillo and Wassarman, 1991). These
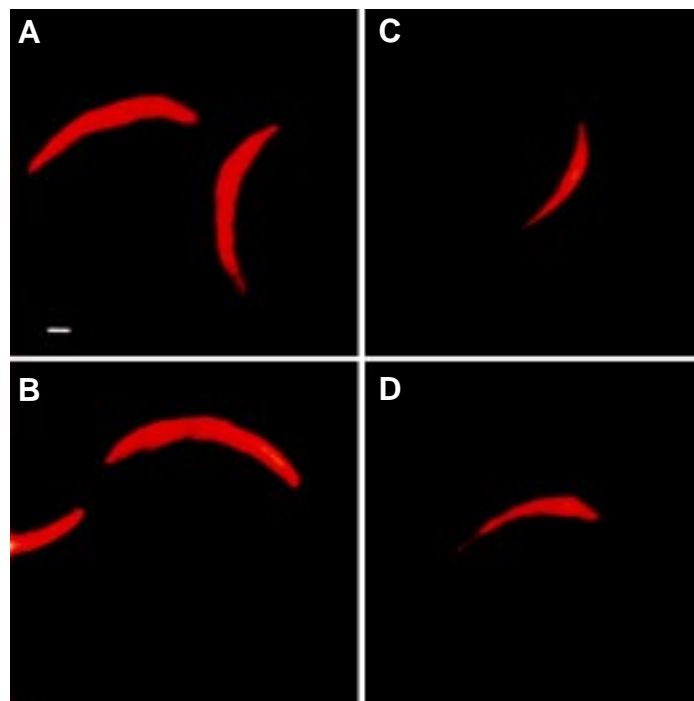

Fig. 5. Laser scanning confocal microscopy (LSCM) of mouse sperm probed with either (A,B) anti-MC101 or (C,D) anti-sp56. *Sperm were obtained, capacitated, fixed, and immunofluorescently labeled, as described in Materials and Methods. The samples shown are representative of the ~1,200 sperm examined by LSCM for each set of experimental conditions (~200 sperm/slide; 2 slides/experiment; 3 individual experiments). A 1 μm bar is shown in Panel A.*

observations by LSCM suggest that sp56 is present in the acrosomal vesicle, as well as at sites outside the acrosome that come in contact with mZP3.

Discussion

A great deal of confusion has been attached to the identification of mammalian sperm EBPs that recognize mZP3 on ovulated eggs (Yanagimachi, 1994; Snell and White, 1996; Wassarman, 1999; Wassarman *et al.,* 2001). A variety of experimental approaches have been employed with sperm from a variety of mammalian species. Several such proteins have been described in some detail and certain of these have characteristics that make them prime candidates (e.g., they specifically bind to mZP3 and are located on the surface of acrosome-intact sperm heads). Among these proteins are β1,4-galactosyltransferase (Miller *et al.,* 1992), zonadhesin (Gao and Garbers, 1998), the spermadhesins (Romero *et al.,* 1997), and sperm protein sp56 (Bookbinder *et al.,* 1995). However, as yet, no single candidate EBP has been fully accepted by workers in the field. This is certainly the case for sperm protein sp56, whose location on sperm has been debated in the literature (Cheng *et al.,* 1994; Foster *et al.,* 1997). If sp56 is located exclusively inside the acrosome, a membrane-bounded vesicle, at first glance it is difficult to envision how it could interact with mZP3 during binding of acrosome-intact sperm to eggs.

Localization of sp56 to the acrosome came about as a result of the cloning of a guinea pig sperm protein, AM67 (Foster *et al.,* 1997). This protein is a secretory component of the guinea pig sperm acrosomal matrix and its primary struc-

Fig. 6. Laser scanning confocal microscopy (LSCM) of mouse sperm probed with anti-mZP3 (green) and anti-sp56 (red). *Sperm were obtained, capacitated, fixed, and immunofluorescently labeled as described in Materials and Methods. Yellow indicates the overloap of the fluorescent signals. The samples shown are representative of the ~1,200 sperm examined by LSCM for each set of experimental conditions (~200 sperm/slide; 2 slides/experiment; 3 individual experiments). A 1 µm bar is shown in Panel A.*

ture it most similar to that of mouse sp56 and the α-subunit of complement component 4-binding proteins from various mammals. However, it was noted that one region of sp56, residues 414-453 (located between Sushi domains 6 and 7; Bookbinder *et al.,* 1995), had no corresponding counterpart in AM67. In this context, it had been reported that sp56 protein and sp56 messenger-RNA are not detectable in guinea pig (or human) sperm by Western immunoblotting and Northern blotting, respectively (Bookbinder *et al.,* 1995). This is an interesting finding since mouse and guinea pig (or human) gametes do not interact with each other (Schmell and Gulyas, 1980; Gulyas and Schmell, 1981), but is very surprising in view of the extensive sequence similarities between AM67 (guinea pig) and sp56 (mouse).

Three pieces of experimental evidence are presented here which suggest that egg mZP3 binds to sperm sp56. (i) Inhibition of sperm binding to eggs by sperm membrane vesicles is relieved by incubating the vesicles in the presence of anti-sp56 (Fig. 2B). Although it could be argued that this is simply a non-specific steric effect, it does suggest that sp56 is associated with the surface of sperm membrane vesicles. How it got there remains a point of conjecture. It should be mentioned that the results are consistent with the finding that sperm fail to bind to eggs incubated in the presence of purified sp56
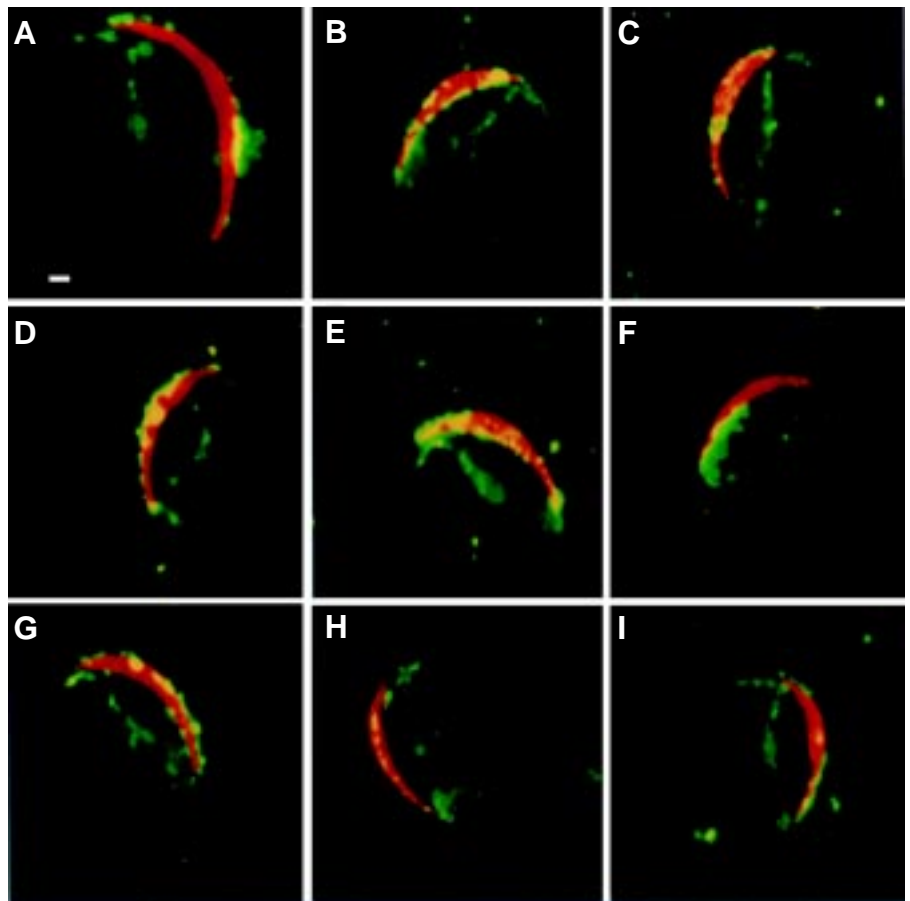
(Bookbinder *et al.,* 1995). (ii) When sperm extracts are incubated in the presence of mZP3, both sp56 and mZP3 are co-immunoprecipitated by either anti-sp56 or anti-mZP3 (Fig. 4). This is strong evidence that the two proteins interact specifically with each other and is consistent with several previous observations (Bleil and Wassarman, 1990; Cheng *et al.,* 1994). (iii) LSCM of sperm incubated in the presence of mZP3 suggests that, while the bulk of sp56 is inside the acrosome, there is overlap of sp56 and mZP3 at the sperm surface (Fig. 6). Once again, it is tempting to conclude from this evidence that mZP3 and sp56 interact specifically with each other.

Several scenarios can be envisioned that place mZP3 in close proximity to sp56, close enough to be chemically crosslinked to each other, even if sp56 were exclusively an acrosomal protein. For example, binding of mZP3 to acrosome-intact sperm is known to trigger the acrosome reaction; i.e., fusion of outer acrosomal membrane and plasma membrane at multiple sites (Bleil and Wassarman, 1983; Yanagimachi, 1994; Florman *et al.,* 1998; Wassarman, 1999; Wassarman *et al.,* 2001). With live sperm, the initial events in this process, following either capacitation or mZP3 binding, may include formation of many small regions of common bilayer between outer acrosomal and plasma membrane, thereby exposing sp56 to the
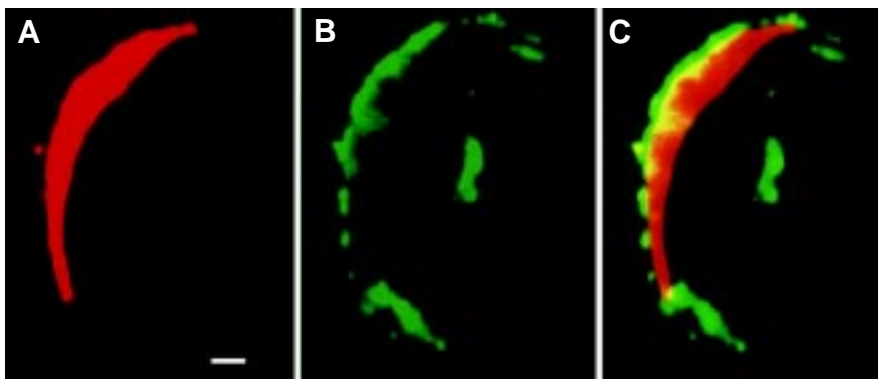
Fig. 7. Laser scanning confocal microscopy (LSCM) of mouse sperm probed with anti-mZP3 (green) and anti-sp56 (red). *Sperm were obtained, capacitated, fixed, and immunofluorescently labeled as described in Materials and Methods and in the legend to Fig. 6. Yellow indicates the overlap of the fluorescence signals. A 1 µm bar is shown in Panel A.*

surface. Such a situation has been reviewed (Monck and Fernandez, 1996) and discussed (Foster *et al.,* 1997) in the literature. Alternatively, either fixation of capacitated sperm or vortexing of capacitated sperm to produce membrane vesicles could trigger initial events of the acrosome reaction and/or other rearrangements, resulting in exposure of sp56 to added mZP3. Since sp56 is reported to be a peripheral, not an integral membrane protein (Cheng *et al.,* 1994), its association with the sperm surface could come about in any number of ways. Finally, it remains a possibility that a fraction of sp56 is normally associated with plasma membrane overlying the sperm head and is accessible to mZP3 binding and affinity-crosslinking (Bleil and Wassarman, 1990). In this context, it remains to be determined why here and in some other reports (Foster *et al.,* 1997; Hardy and Mobbs, 1999), but not in all reports (Cheng *et al.,* 1994; Suzuki-Toyota *et al.,* 1995), anti-sp56 fails to be detected on live sperm.

In conclusion, several pieces of experimental evidence described here strongly suggest that egg mZP3 binds to sperm sp56, despite the fact that the bulk of antigen recognized by anti-sp56 is present in the sperm acrosomal vesicle and is not detectable on live sperm. These apparently contradictory findings can be explained by movement of sp56 to the sperm surface as a result of capacitation, initiation of the acrosome reaction, and/or some other stimulus. Alternatively, a small fraction of sp56, difficult to detect on live sperm, may be associated with the outer surface of sperm plasma membrane.

Materials and Methods

Collection and culture of gametes and embryos

Ovulated eggs and two-cell embryos were collected 6-10 h after ovulation and 32-36 h after fertilization, respectively, from oviducts of superovulated female Swiss albino mice, 4-8 weeks-of-age (CD-1; Charles River Breeding Labs, Wilmington, MA), essentially as previously described (Litscher and Wassarman, 1999). Eggs and embryos were cultured in Earle's modified medium M199 (Gibco BRL, Rockville, MD) containing 25 mM Hepes, pH 7.3, supplemented with BSA (4 mg/ml; Sigma, fraction V) and pyruvate (30 µg/ml) (M199-M). Eggs and embryos were treated with hyaluronidase (1 mg/ml) to remove cumulus cells and fixed in 30 mM sodium phosphate, pH 7.2, containing 150 mM NaCl, PVP-40 (4 mg/ml), 0.02% sodium azide (PBS/PVP-40), and 1% formaldehyde. Fixed eggs were stored for up to 1 week at 4°C in M199 supplemented with 50 mM Tris-HCl, pH 7.5, containing PVP-40 (4 mg/ml) and 0.02% sodium azide. Fixed embryos were stored in the same manner for up to 1 month. Sperm were collected from caudae epididymae excised from mature CD-1 male mice, placed in M199-M containing 4 mM

EGTA, and pelleted by low speed centrifugation. Fresh M199-M was added to the pellet and sperm were permitted to swim into the medium. Sperm were capacitated in M199-M for 1 h at 37°C prior to use.

Preparation of sperm membrane vesicles

Sperm membrane vesicles were prepared essentially as previously described (Bunch and Saling, 1991; Kalab *et al.,* 1994). Uncapacitated caudal epididymal sperm were collected in 1 ml of ice-cold 20 mM Tris-HCl, pH 7.1, containing 130 mM NaCl and 2 mM EGTA (TN-EGTA). Sperm were suspended in 13-15 ml of ice-cold TN-EGTA and contaminating pieces of tissue were removed by centrifugation at 100 x g for 1 min at room temperature (RT). Sperm were then subjected to centrifugation at 800 x g for 10 min at 4 °C, resuspended in 2 ml of TN-EGTA, and their motility assessed by light microscopy. Although sperm motility was reduced at 4 °C, at least 60% of the cells were highly motile. Resuspended cells were placed in a 15 ml plastic tube and subjected to vortex treatment by a Genie 2 vortex at setting-6 for ~150 sec. The vortexed suspension was pelleted at 3,000 x g for 10 min at 4°C and the supernatant recovered. The sperm pellet was resuspended in 2 ml TN-EGTA and centrifuged at 3,000 x g again. The supernatants were combined and residual sperm removed by three centrifugation steps. The supernatant was then spun at 105,000 x g in a Beckman 50 Ti fixed-angle rotor for 1 h at 4°C. The high-speed pellet was then resuspended in PBS for use in experiments.

Isolation of sperm heads and tails

Mouse sperm were decapitated by trypsin treatment, and heads and tails were fractionated by isopycnic centrifugation on a metrizamide gradient, essentially as previously described (Bellvé *et al.,* 1993). Briefly, sperm were collected in PBS, adjusted to ~10^7 cells/ml, and trypsin added (100 µg/ml). Sperm were then homogenized in a loose-fitting Dounce homogenizer for 1-2 min at RT, until heads and tails were separated. A 10-fold excess of soybean trypsin inhibitor was added immediately and samples were placed on ice to inhibit further tryptic activity. Heads and tails were washed 3-times in 50 mM Tris-HCl, pH 7.4, containing protease inhibitors (PMSF, 1 mM; EGTA, 0.5 mM; aprotinin, 1 µg/ml; leupeptin, 1 µg/ml; pepstatin, 1 µg/ml; and antipain, 5 µg/ml) at 4 °C. The preparation was then subjected to centrifugation on a 40-60% metrizamide gradient, prepared as previously described (Bellvé, 1993), in an SW41 Ti rotor at 76,000 x g for ~15 h at 4 °C. Sperm heads and tails formed distinct bands that were recovered by puncturing the tubes with a syringe.

Sperm binding assay (competition assay)

Binding assays were carried out in M199-M at 37°C in a humidified atmosphere of 5% CO_2 in air, essentially as previously described (Bleil and Wassarman, 1980; Florman and Wassarman, 1985; Litscher and Wassarman, 1999). Ovulated eggs (10-15/assay) and two-cell embryos (2-3/assay) were washed through at least 3 drops of M199-M prior to use. In some cases, eggs and embryos were incubated in 10 µl of M199-M in the presence of various amounts of sperm membrane vesicles for 15 min. Capacitated sperm (10 µl) were then added to the cells and the incubation continued for another 30 min. In other cases, sperm were incubated in 10 µl of M199-M in the presence of various amounts of antibodies for 15 min. Eggs and embryos in 10 µl of M199-M were then added to the sperm and the incubation continued for another 30 min. At the end of the final incubation, eggs and embryos were washed with M199-M using mouth-operated, glass micropipettes, cells were fixed with 1% formaldehyde in PBS/PVP-40, and the number of sperm bound to eggs determined by dark-field microscopy.

Acrosome reaction assay

The status of the mouse sperm acrosome was assessed using the previously described *Pisum sativum* agglutinin (PSA) staining technique (Mendoza *et al.,* 1992; Margalit *et al.,* 1997). Sperm were dried on glass

slides, fixed in methanol for 30 sec, and incubated in blocking buffer (PBS containing 1% BSA) for 10 min. Slides were treated with biotin-conjugated PSA (50 μg/ml) in blocking buffer for 10 min at RT, washed with PBS, and then with peroxidase-conjugated avidin (1:400 dilution) in blocking buffer for 10 min at RT. Color was developed by addition of substrate (AEC Kit; Zymed Labs, San Francisco, CA) for 10 min at RT and cells were counterstained with hematoxylin for 3 min at RT. Slides were then washed, dried, mounted, and examined by light microscopy. An average of 200 sperm were assessed per slide. Sperm possessing a distinct red cap were assessed as acrosome-intact.

Western immunoblot analysis

Samples were subjected to SDS-PAGE under non-reducing conditions, transfered to nitrocellulose, and incubated overnight at 4°C in the presence of primary antibody (either mouse anti-sp56, ~25 μg/ml or rabbit anti-mZP3, 1:500 dilution; antibodies described below). Blots were then incubated for 1 h at RT in the presence of either horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,500 dilution; BioRad, Melville, NY) and processed for ECL or alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2,500 dilution; BioRad) and NBT/BCIP (Gibco/BRL).

Immunoprecipitation analysis

Capacitated sperm from 4-5 male mice were extracted in 200 μl of lysis buffer (50 mM Tris-HCl, pH 7.2, containing 0.1% Triton X-100, 150 mM NaCl, 10% glycerol, and 2 mM EDTA), supplemented with protease inhibitors (aprotinin, 10 μg/ml; leupeptin, 10 μg/ml; pepstatin, 1 μg/ml; antipain, 5 μg/ml; and PMSF, 1 mM), for 1 h on ice. Insoluble material was removed by centrifugation at 14,000 rpm for 10 min at 4°C and the clarified lysate was diluted by addition of an equal volume of lysis buffer lacking detergent. Half the lysate was incubated alone and the other half in the presence of purified mZP3 (~2 μg) for 30 min at 4°C. Both lysates were immuoprecipitated using either anti-mZP3 or anti-sp56 (antibodies described below) for 1, 4, or 24 h at 4°C. Protein A-agarose beads (Boehringer Mannheim, Indianapolis, IN) were added (1:20 ratio) and the samples were rotated for 2 h at 4°C. Beads were collected by centrifugation, washed with lysis buffer lacking detergent, and the supernatant set aside. Beads and supernatant were boiled for 5 min with non-reducing sample buffer and subjected to SDS-PAGE and Western immunoblotting (described above).

Electron microscopy of membrane vesicles

Freshly prepared sperm membrane vesicles were resuspended in 2% gluteraldehyde made up in PBS, centrifuged at 105,000 x g for 1 h in a Beckman 50 Ti fixed-angle rotor, and the pellet was incubated in 2% gluteraldehyde overnight at 4°C. The pellet was transfered through several PBS washes, post-fixed for 30 min in 1% osmium tetroxide made up in PBS, washed, stained for 1 h with 1% uranyl acetate, and dehydrated through a graded series of ethanol (50-100%). The pellet was embedded in Epon 812, and thin sections were cut and examined by electron microscopy.

Laser scanning confocal microscopy (LSCM) of sperm

Capacitated mouse sperm suspensions were placed on glass slides, fixed in PBS containing 3.7% paraformaldehyde for up to 30 min at RT, washed 3-times in PBS, and incubated in blocking buffer (PBS/PVP-40 containing 2% BSA) for 30 min at RT. In experiments involving purified mZP3, 100 μl of sperm were incubated with 1.5 μg of mZP3 in M199-M for 30 min at 37°C and washed with PBS/PVP-40 prior to fixation. For immunofluorescent staining, slides were incubated with primary antibodies: (i) MC101, a mouse monoclonal antibody directed against a ~155 kDa M_r sperm acrosomal protein (1:250 dilution; provided by Dr. Tadashi Baba); (ii) 7C5, a mouse monoclonal antibody directed against sperm protein sp56 (~40 μg/ml; purchased from QED Biologicals, La Jolla, CA or provided by Dr. Jeffrey Bleil); and (iii) anti-mZP3, a rabbit polyclonal antibody directed against mouse mZP3 (1:1,000 dilution; Pocono Rabbit Farms, Canadensis, PA). Slides were washed with blocking buffer and treated with secondary antibodies; Texas Red-conjugated goat anti-mouse IgG and/or FITC-conjugated goat anti-rabbit IgG (1:50 dilution; Molecular Probes, Eugene,

OR) for 1 h at RT. After washing, slides were incubated in equilibration buffer for 15 min and mounted using Slow Fade Light Antifade Kit (Molecular Probes). Slides were examined using a Leica inverted LSCM, and images were processed by Adobe Photoshop software and printed with a color dye sublimation printer.

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