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Equatorial Segment Protein (ESP) Is a Human Alloantigen Involved in Sperm-Egg Binding and Fusion

M. J. Wolkowicz, **L. Digilio**, **K. Klotz**, **J. Shetty**, **C. J. Flickinger**, and **J. C. Herr**
Center for Research in Contraceptive and Reproductive Health, University of Virginia, School of Medicine, Charlottesville, Virginia

Abstract

The equatorial segment of the sperm head is known to play a role in fertilization; however, the specific sperm molecules contributing to the integrity of the equatorial segment and in binding and fusion at the oolemma remain incomplete. Moreover, identification of molecular mediators of fertilization that are also immunogenic in humans is predicted to advance both the diagnosis and treatment of immune infertility. We previously reported the cloning of Equatorial Segment Protein (ESP), a protein localized to the equatorial segment of ejaculated human sperm. ESP is a biomarker for a subcompartment of the acrosomal matrix that can be traced through all stages of acrosome biogenesis (Wolkowicz et al, 2003). In the present study, ESP immunoreacted on Western blots with 4 (27%) of 15 antisperm antibody (ASA)–positive serum samples from infertile male patients and 2 (40%) of 5 ASA-positive female sera. Immunofluorescent studies revealed ESP in the equatorial segment of 89% of acrosome-reacted sperm. ESP persisted as a defined equatorial segment band on 100% of sperm tightly bound to the oolemma of hamster eggs. Antisera to recombinant human ESP inhibited both oolemmal binding and fusion of human sperm in the hamster egg penetration assay. The results indicate that ESP is a human alloantigen involved in sperm-egg binding and fusion. Defined recombinant sperm immunogens, such as ESP, may offer opportunities for differential diagnosis of immune infertility.

Keywords

Antisperm antibodies; sperm alloantigens; immune infertility; spermatogenesis; acrosome; fertilization

Naturally occurring antisperm antibodies (ASAs) have been associated with some cases of otherwise unexplained infertility in women (Rumke and Hellinga, 1959), and they rise following vasectomy in a high proportion of men (Alexander and Anderson, 1979), with ASA titers often persisting for years (Rose and Lucas, 1979). ASAs are believed to cause infertility by inhibiting sperm motility, sperm penetration of cervical mucus, and capacitation; initiating the acrosome reaction prematurely; activating the complement cascade leading to sperm lysis; or coating sperm and blocking steps in the fertilization cascade. Knowledge of the identities of the specific sperm proteins that elicit the production of ASAs would lead to a better understanding of the mechanism of immunologic infertility. Additionally, identification of those infertile patients possessing ASAs to antigens known to relate to be involved in fertilization might lead to more precise diagnoses.

Correspondence to: John C. Herr, Department of Cell Biology, University of Virginia Health System School of Medicine, P.O. Box 800732, University of Virginia, Charlottesville, VA 22908 (jch7k@virginia.edu).

Recently, in conjunction with a comprehensive human sperm proteome (Naaby-Hansen et al, 2002), two-dimensional (2D) Western blots were employed to assess the concanavalin A (Con-A) binding capabilities of human sperm proteins. This led to the discovery of a novel 349–amino acid Con-A binding protein, Equatorial Segment Protein (ESP; Wolkowicz et al, 2003). ESP localizes to the equatorial segment of ejaculated human sperm and is encoded by a 2-exon gene (*SP-ESP*) located on chromosome 15 at q22. Northern blot analysis demonstrated that ESP is abundantly expressed in the testis and is also detected as an expressed sequence tag transcript (EST) in placenta. However, immunocytochemical staining of placental tissue failed to detect the protein. Immunofluorescent observations on human sperm using polyclonal antibodies to recombinant human ESP (rec-h-ESP) revealed that ESP delineates an equatorial segment domain during early acrosome biogenesis. ESP first appeared in the nascent acrosomal vesicle in early round spermatids and subsequently segregated to the periphery of the expanding acrosomal vesicle, thereby defining a peripheral equatorial segment compartment within flattened acrosomal vesicles, and it localized as well in the acrosomes of early and late cap phase, elongating, and mature spermatids. Thus, ESP serves as a marker for the specification of the equatorial segment as early as the Golgi phase of acrosome biogenesis and allows the equatorial segment to be traced through all subsequent phases of acrosomal biogenesis.

The equatorial segment of the acrosome is of considerable functional importance to fertilization because it 1) remains intact following the acrosome reaction, 2) underlies the domain of the plasma membrane involved in fusion with the egg membrane, and 3) is the site where breakdown of the sperm nuclear envelope is initiated after fertilization (Yanagimachi and Noda, 1970). The present immunologic studies focused on two main questions: 1) Is ESP immunogenic in humans? and 2) Is ESP involved in fertilization events mediated by the equatorial segment? Western blots (1D and 2D) of human sperm (Naaby-Hansen et al, 1997) were examined with ASA-positive sera from infertile patients (Shetty et al, 1999), and the immunoreactive proteins were compared to blots of sera from fertile individuals, revealing that ESP is alloantigenic. Sera from patients with antisperm antibodies also recognized recombinant ESP. ESP persisted on capacitated, acrosome-reacted human sperm as well as on sperm bound to hamster oocytes, and in accord with this retention of ESP, antisera raised to recombinant ESP inhibited the binding and fusion of human sperm in the hamster egg penetration assay. The results indicate that ESP is involved in key events of fertilization and may be useful in the diagnosis of antibody-mediated infertility and in understanding its underlying mechanisms.

Materials and Methods

Protein Electrophoresis and Western Blotting

Human sperm proteins for 2D gel electrophoresis were solubilized and separated on 2D polyacrylamide gels as previously described by Naaby-Hansen et al (1997). Standard 1D sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Colligan et al, 1995) for recombinant proteins and prokaryotic cell lysates was performed on a 16 × 18 cm gel electrophoresis apparatus (Bio-Rad, Hercules, California) with 0.75-mm spacers and 12% polyacrylamide separating gels. Protein or cellular samples were either suspended in standard Laemmli buffer (Laemmli, 1970) and boiled for 10 minutes before addition to the gel or treated with iodoacetic acid by the procedure of Crestfield et al (1963). *Escherichia coli* lysates consisted of 1 optical density (OD)-mL of bacterial culture, pelleted before extraction in Laemmli loading buffer.

Electrophoretic transfer of separated proteins to nitrocellulose membranes was performed in transblot buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% methanol) for 1 A/h. The membranes were either used intact for 2D gels or, in the case of standard 1D protein gels, cut into strips, blocked with 5% fat-free dry milk in phosphate-buffered saline–Tween 20 (1× PBS [pH 7.4], 0.05% Tween 20), and then incubated in rat antisera to ESP for 1 hour at 22°C.

Immunodetection was performed with horseradish peroxidase–conjugated goat anti-rat IgG (Jackson ImmunoResearch, West Grove, Pennsylvania) at a 1:5000 dilution in blocking buffer and visualized with diaminobenzidine (Sigma, St Louis, Missouri) in H_2O_2 or TMB (Kierkegard and Perry Laboratories, Gaithersburg, MD). Rat monospecifc polyclonal antisera to ESP was prepared as previously described (Wolkowicz et al, 2003) by cloning the human ESP cDNA, expressing the recombinant protein in *E coli*, purifying the protein, injecting the material into virgin female Lewis rats, and collecting the immune sera. Western blots of 2D separated human proteins also were screened with a 1:2000 dilution of sera from infertile patients containing ASAs (Shetty et al, 1999) as determined by the immunobead binding test (IBT; Bronson and Tung, 1992) as well as with fertile control sera (1:2000 dilution). Bound ASAs were detected by incubation of thrice-washed blots for 1 hour at 20°C with secondary enzyme-conjugated goat anti-human immunoglobulin G (IgG) + IgM antibodies (Jackson ImmunoResearch) diluted 1:5000 in PBS containing 0.05% Tween 20. Horseradish peroxidase conjugates were visualized by enhanced chemiluminescence using the manufacturer's protocol (Amersham, Buckinghamshire, United Kingdom). Sequencing by LC-MS analysis was performed by the W. M. Keck Foundation Center for Biomedical Mass Spectrometry at the University of Virginia on an LC-Q apparatus (Finnigan, Austin, Texas) according to the manufacturer's instructions and as described previously (Wolkowicz et al, 2002).

Serum From Infertile Patients

Serum samples were obtained from infertile men and women with unexplained infertility. The infertile subjects did not have any previously diagnosed hormonal, infective, or physical causes for their infertility. The male subjects had not undergone vasectomy. The ASAs in the serum samples from infertile subjects were detected by direct or indirect immunobead binding as previously described (Bronson and Tung, 1992). All the serum and sperm samples were tested for the presence of IgG-, IgM-, and IgA-specific ASAs, and included samples from 15 infertile male subjects, 5 infertile female subjects, 5 fertile male subjects, and 5 fertile female subjects. All of the control fertile subjects were individuals with proven fertility and were ASA negative based on the immunobead test. Serum samples from infertile subjects with more than 60% of the spermatozoa binding beads, indicative of IgG- and/or IgM-specific ASAs, were used for 2D Western blotting. All of the selected samples contained antibodies directed against the sperm head or the entire spermatozoa.

Hamster Egg Penetration Xeno-Assay

Sperm-egg interaction was assessed in a binding and fusion assay using zona-free hamster eggs. Biggers, Whitten and Whittingham (BWW) media (2 mL; Irvine Scientific, Santa Ana, California) containing 5 mg/mL human serum albumin (HSA) was underlayed with human sperm ejaculate, and the sperm were allowed to swim up for 1.5–2 hours. The swim-up sperm were collected, and 8 mL BWW and 5 mg/mL HSA were added before centrifugation at 600 × *g* for 8 minutes at 22°C. This wash was repeated before the pellet was suspended in 50 μL BWW containing 30 mg/mL HSA. Sperm were counted and incubated overnight in BWW and 30 mg/mL HSA at a concentration of 2×10^6 sperm/mL. Hamster eggs were collected by injecting female hamsters intraperitoneally with 30 IU pregnant mare serum gonadotropin (PMSG), followed 72 hours later by 30 IU human chorionic gonadotropin (hCG). After 14–16 hours, the hamsters were sacrificed, oviducts were collected in BWW media containing 5 mg/mL HSA, and cumulus cells were removed with 1 mg/mL hyaluronidase. Eggs were washed and zona pellucidae removed with 1 mg/mL trypsin before final thorough washing and incubation. Diluted sperm were incubated with preimmune or immune sera to ESP (1:10 and 1:50 dilutions were tested) in paraffin oil–covered microdrops for 1 hour before addition of the collected hamster eggs. Preimmune and immune rat sera against the intra-acrosomal protein C113 were used as a second control at 1:10, 1:50, and 1:100 dilutions.

Gametes were coincubated for 3 hours, and sperm and egg binding was assessed after washing unbound and loosely bound sperm by serial passage through 5 (50 μL) wash drops. Eggs then were stained by 5–15 seconds of exposure to 1 mM acridine orange/3% DMSO in bovine serum albumin (BSA)/BWW (30 mg/mL), washed through 4 (50 μL) wash drops, and mounted under 22-mm coverslips. Under ultraviolet illumination, unexpanded heads of oolemma-adherent sperm were counted, as were sperm that had penetrated the ooplasm and exhibited expanded green heads. All in vitro assays were repeated 3 times. Data for experimental and control groups were reported as means ± SD. Student's *t* test was used to identify differences between treated and control samples in the number of bound or fused spermatozoa. $P < .05$ was considered to be statistically significant.

Immunostaining of Human Sperm/Hamster Eggs

Mature ova were collected from the oviducts of superovulated hamsters 15 to 17 hours after intraperitoneal injection of hCG. The cumulus cells were removed by treating the eggs for 3 minutes with 0.1% hyaluronidase in BWW media containing 5 mg/mL HSA. The zonae pellucidae were removed with 0.1% trypsin treatment for 2–3 minutes. The washed zona-free eggs were placed in BWW medium in 100-μL drops under mineral oil. Human semen from healthy donors was obtained and allowed to liquefy for 30 minutes at 22°C. Semen (500 μL) was underlain with 2 mL BWW media containing 5 mg/mL HAS, and sperm were allowed to swim up for 2 hours at 37°C and 5% CO_2. Following the swim-up period, sperm were removed and added to 8 mL BWW containing 5 mg/mL HSA. The sample was washed 2 times by centrifugation at 600 × *g* for 8 minutes at 22°C. The pellet then was resuspended in BWW containing 30 mg/mL HSA. Sperm were capacitated overnight at 20×10^6 sperm cells/mL at 37°C and 5% CO_2. The sample was diluted to 2×10^6 sperm/mL in 20-mL drops, and 10 zona-free eggs were added to each drop. Gametes were coincubated for 3 hours at 37°C and 5% CO_2. Subsequently, the eggs were washed gently in BWW medium and fixed in 4% paraformaldehyde for 20 minutes at 22°C. Following fixation, eggs were incubated overnight with 15 μg/mL of 3C6 IgG overnight at 4°C. The 3C6 is a monoclonal antibody that we prepared that recognizes the various isoforms of ESP essentially as demonstrated and described in Wolkowicz et al (2003). Eggs then were washed and incubated with a 1:100 dilution of Texas Red–conjugated donkey anti-mouse IgG for 1 hour at room temperature. The eggs then were washed extensively (4 × 10-minute washes), placed in 0.4 mg/mL RNase in PBS with 1% BSA for 30 minutes, and stained with 20 nM Sytox (Molecular Probes, Eugene, Oregon) for 10 minutes. The eggs were again extensively washed, placed in slow fade (Molecular Probes) equilibration media for approximately 1 minute, and then mounted in slow fade mounting media. Scanning confocal microscopy images were obtained on a Zeiss 410 Axiovert 100 microsystems LSM confocal microscope (Carl Zeiss GmbH, Jena, Germany). Attenuation, contrast, brightness and pinhole aperture remained constant. Four-second scans were averaged 4 times per line using a × 63 oil lens.

Dual Staining of Acrosome-Reacted Sperm

To determine the relationship between acrosomal status and the presence of ESP, capacitated, acrosome-reacted sperm were dually stained with PSA lectin, to assess acrosome reactivity, and with antibody to ESP. Sperm were prepared by modifying the procedure of Garcia and Meisel (1999). Briefly, liquefied ejaculates were pooled and 0.25 mL semen was layered under 2 mL BWW medium with 34 mM $NaHCO_3$ in 10 × 75 mm round-bottom tubes. Sperm were allowed to swim up into the medium for 1 hour at 37°C in a 5% CO_2 atmosphere. Swim-up sperm were aspirated and washed 1× in BWW by centrifugation at 700 × *g* for 10 minutes. Sperm were resuspended in BWW with 30 mg/mL fatty acid–free BSA (Sigma). Aliquots of these “noncapacitated” sperm were washed twice in PBS at 600 × *g* for 8 minutes and then spotted on microscope slides and allowed to air dry. The remaining sperm were allowed to capacitate for 6 hours in Petri dishes in BWW medium with 30 mg/mL BSA and at 37°C with

5% CO_2 with penicillin (100 U/mL) and streptomycin (100 μg/mL; Gibco/BRL, Rockville, Maryland) present. To one group of capacitated sperm, a 1:500 dilution of 2.5 mM stock progesterone (Sigma) solution in ethanol was added for a final concentration of 5 μM progesterone. A control group received an equal volume of ethanol alone at a 1:500 dilution. After incubation for 20 minutes at 37°C in 5% CO_2, both groups were washed twice in PBS at 600 × *g* for 8 minutes and then spotted on microscope slides.

Previously, live staining of human sperm with the α–rec-hESP polyclonal serum demonstrated no reactivity, indicating that the ESP protein was not present on the surface of the sperm (Wolkowicz et al, 2003). Acrosome-reacted sperm were detected by adapting the procedure of Cross et al (1986). Air-dried sperm on microscope slides were blocked for 15 minutes with 1% BSA in PBS and incubated for 1 hour at 22°C with undiluted supernatant from a murine cell line (3C6), producing a monoclonal antibody to ESP. Another slide was incubated with 1% BSA in PBS as a negative control. After washing with PBS, the cells were incubated for 1 hour at 22°C with TRITC-conjugated $F(ab)_2$ fragments of donkey anti-mouse IgG (Jackson ImmunoResearch) diluted 1:50 in PBS with 1% BSA. After washing with PBS, the sperm were fixed for 15 minutes with 4% paraformaldehyde in PBS, washed with PBS, and incubated with 50 μg/mL FITC-conjugated PSA lectin (Sigma). The dual-stained cells were observed with a Zeiss Axiovert 200 microscope equipped for epifluorescence. Photos were taken of identical fields with fluorescein and rhodamine excitation filters using a Diagnostic Instruments 4.1 digital camera and Spot Insight software (Diagnostic Instruments Inc, Sterling Heights, Michigan). Sperm were scored visually as acrosome intact or acrosome reacted according to the degree of staining with PSA lectin, and the same sperm were scored for the presence or absence of ESP immunoreactivity.

Results

Screening of 2D Western Blots for ASA-Positive Sperm Antigens

To investigate the immunoreactivity of proteins possibly involved in human infertility, 2D Western blots of human sperm proteins were probed with a pool of ASA-positive sera from 5 infertile male individuals or with sera from fertile donors (Figure 1B and A, respectively). Proteins that were immunoreactive with both the male fertile and ASA-positive infertile sera were noted such as a broad pI region around 90 kDa, a series of proteins between 60 and 90 kDa, pI 5.5 to 6.0, as well as others. Microsequences obtained from proteins with a broad pI range around 90 kDa previously yielded fibrous sheath proteins present in the sperm tail (Naaby-Hansen et al, 2002). As tissue-specific proteins from the sperm's unique cytoskeleton, these proteins demonstrate alloantigenic responses in both the fertile and infertile sera of both males and females.

In addition to common spots immunoreactive with sera from both fertile and infertile males and females, the ASA-positive infertile male individuals recognized several alloantigenic proteins (Figure 1B) that were not stained on immunoblots reacted with control sera from fertile males (Figure 1A). An oblique train of spots at approximately 36–38 kDa and 5.1 pI, designated C7 and C8, was bound by ASA-positive infertile male sera (Figure 1B, arrows) but was not recognized by the fertile male sera pool (Figure 1A). In all, 15 sera from infertile men were assayed separately by 2D Western blots, and 4 (26.7%) of the 15 recognized these same spots with identical patterns on Western blots. Concomitantly with the above experiments, 5 ASA-positive sera from 5 infertile female individuals as well as sera from 5 fertile females also were assayed in a similar manner vs human sperm proteins, and 2 of the samples recognized C7 and C8 spots (data not shown). Both infertile and fertile female sera recognized some of the same patterns of proteins and ubiquitous areas as the male sera to varying degrees (data not shown). However, the area important to the present study was recognized by a pool of infertile female sera and not recognized at all by a fertile sera pool.

Probing 2D gels with Con-A (Figure 2) also proved useful in identifying the precise location of ESP on 2D gels. In Figure 2, the Western blot was first stained with Ponceau S to ascertain both the efficiency of transfer and to yield a traceable pattern of sperm proteins for subsequent comparison (Figure 2A). After removal of the stain, the blot was incubated with horseradish peroxidase–conjugated Con-A to reveal glycosylated human sperm proteins (Figure 2B). Con-A stains a limited subset of proteins, including the area at approximately 36–38 kDa and 5.1 pI, that encompasses the area and configuration revealed by the probing with ASA-positive sera. Microsequencing of the spots indicated in Figure 1B at the top and bottom of this protein train (indicated by C7 and C8) yielded microsequences (AATVFNTLK, LYEYLDLK, SLALAAAAEHK) contained within the recently cloned sperm protein ESP (Wolkowicz et al, 2003), thus confirming by mass spectometry that the glycosylated protein spots were ESP.

Both Polyclonal and ASA-Positive Sera Recognize Recombinant ESP

To confirm that ESP was in fact recognized by the infertile sera, rec-h-ESP (Wolkowicz et al, 2003) was separated by gel electrophoresis, Western blotted, and probed with both infertile sera and sera previously raised to h-ESP (Figure 3, lane 1). The purified rec-h-ESP protein had previously been used to generate a polyclonal serum in rats (Wolkowicz et al, 2003). Preimmune rat sera failed to recognize rec-h-ESP (Figure 3, lane 2), whereas rats injected with rec-h-ESP showed a robust response to the purified protein (Figure 3, lane 3). Blots of rec-h-ESP incubated with pools of both human ASA-negative fertile (Figure 3, lane 4) showed no reactivity, whereas ASA-positive infertile male sera (Figure 3, lane 5) reacted with a single band that comigrated with the purified control recombinant material and immunoreactive rec-h-ESP (Figure 3, lanes 1 and 3, respectively). That ASA-positive human infertile male serum reacted positively with the rec-h-ESP protein confirmed that rec-h-ESP retained epitopes recognized by human ASAs. Taken together, the preceding results demonstrate that ESP is a sperm alloantigen in humans.

Acrosome Reaction and In Vitro Fertilization

A substantial amount of literature points to the equatorial segment's involvement in sperm-egg adhesion and membrane fusion (Yanagimachi and Noda, 1970; Yanagimachi, 1994; Arts et al, 1994, 1997). To test the possible role of ESP in these events, the fate of ESP was studied after in vitro fertilization. Capacitated human sperm and zona-free hamster eggs were incubated with ESP antiserum, and the numbers of sperm bound and those fused were determined and compared to control samples incubated with preimmune serum (Figure 4). The results indicate a 42% reduction in sperm binding (Figure 4A) in the presence of antiserum to ESP ($P \leq .05$). More importantly, we observed a 68% inhibition in the fusion of sperm with eggs ($P \leq .05$; Figure 4B). When the data on fused sperm were expressed as a ratio of fused/bound + fused to eliminate the effect of binding on fusion (Figure 4C), it was noted that there was a statistically significant 52% reduction in fusion ($P \leq .01$). No effect on either sperm binding or fusion was observed with the rat antiserum to the intra-acrosomal protein C113 at any dilution.

The observation that antibody to ESP interfered with sperm-egg binding and fusion led to a prediction that ESP would persist to some degree on acrosome-reacted sperm. Since fertilization is a 2-step process of sequential sperm binding and then fusion of the equatorial segment, if ESP plays a role in fusion, then it should be retained and exposed in acrosome-reacted sperm. Dual staining with PSA lectin and rat α–rec-h-ESP antibodies was performed as illustrated in Figure 5. The loss of the PSA-positive principal segment of the acrosome and retention of a PSA-positive equatorial segment was observed (Figure 5), indicating acrosome-reacted sperm also stained positively for, and retained, ESP (Figure 5). Sperm cells from this dual staining procedure were counted to determine the numbers of acrosome-reacted sperm and those acrosome-reacted sperm that retained ESP staining (Table). A total of 17.8% of human sperm underwent spontaneous or induced acrosome reaction when treated with

progesterone, and 89% of these were positive for ESP staining in an equatorial band. These results indicate that a significant population of sperm retain ESP in their equatorial segment after the progesterone-induced acrosome reaction. This finding is in concert with the prediction from the observation of 68% inhibition of sperm-egg fusion that ESP is retained and exposed on acrosome-reacted sperm.

Direct Observation of ESP in Bound Sperm

The finding that antibody to the ESP reduced both sperm-egg binding and fusion (Figure 4) and that a subpopulation of sperm retained ESP in the equatorial segment after an in vitro acrosome reaction (Table) led us to a direct observation of the fate of ESP on sperm already bound to the egg (Figure 6). Capacitated human sperm and hamster eggs were incubated together for 3 hours, washed thoroughly, fixed, and double stained. Examination by scanning confocal microscopy of the egg in Figure 6, for example, demonstrated 6 bound sperm in different planes of focus, as depicted in Figure 6, 1A through D and 2A through D. Sytox was employed to highlight condensed chromatin in the sperm head (Figure 6, 1A and 2A; green fluorescence). All 6 sperm bound to the oolemma (eg, Figure 6, 1A and 2A), showed immunofluorescent equatorial bands when stained with the α–rec-h-ESP sera (Figure 6, 1B and 2B; red immunofluorescence). Overlaying the 2 sets of fluorescent images onto the phase microscopy image (Figure 6, panels 1D and 2D) demonstrates a yellow fluorescent band at the approximate location of the equatorial segment in each case. In addition, attached sperm stained with the control preimmune sera were negative (data not shown). Of 78 sperm observed on 10 eggs, 100% were ESP positive, indicating that ESP is retained after tight binding of human sperm to hamster eggs.

Discussion

ESP as a Mediator of Sperm-Egg Binding and Fusion

Early studies by Yanagimachi and Noda (1970) led to the hypothesis that the equatorial segment is an acrosomal domain specifically involved in binding and fusion of sperm with the oolemma (Yanagimachi, 1994). Subsequently, correlative evidence has supported this long-held paradigm. Indeed, addition of equatorial segment–positive monoclonal antibodies, such as the anti-hamster M1 (Noor and Moore, 1999) or the anti-murine MN9 (Toshimori et al, 1992), to in vitro bioassays involving intact and zona-free eggs inhibits the binding and fusion steps of fertilization. In the experiments presented here, the use of polyclonal antiserum generated against ESP revealed a significant inhibition of both sperm binding to and fusion with the egg membrane in comparison with the preimmune serum in the hamster egg penetration assay. Additionally, although a significant decrease in both the binding and fusion was noted, the greater reduction in fusion independent of binding suggests that ESP may be particularly important for this latter step.

It is particularly interesting that all the human sperm that tightly bound to the hamster oocyte surface were ESP positive. One tentative interpretation of this observation may be that an intact equatorial segment may be required to achieve tight binding. Now that a probe specific for the human equatorial segment is available, it may be important to extend such observations to human oocytes.

Regions of ESP protein sequence with homology to other molecules (Figure 7) suggest functionalities that relate both to the location of ESP in the equatorial segment (Wolkowicz et al, 2003) and to binding and fusion events. ESP's N-terminal portion is homologous to the cytolysin family of proteins, which function in oligomerization, membrane insertion, cholesterol binding, and pore formation (Palmer, 2001). A C-terminal domain in ESP homologous to a bacterial type II membrane-binding protein is overlaid on top of a portion of

the osteoglycin domain. Type II membrane-binding proteins are used by bacteria in attaching their chromosome to the interior wall of the cell. Lastly, the C-terminus of ESP is homologous to a 68–amino acid region of osteoglycin. Osteoglycin belongs to a large family of leucine-rich repeat proteoglycans that includes decorin, keratocan, fibromodulin, and biglycan (Matsushima et al, 2000) that act through protein-protein interactions and appear to mediate such diverse cellular processes as signal transduction, cell adhesion, and recombination. In fact, ESP does contain a conserved serine residue (ESP amino acid 306) thought to enable covalent cross linking (osteoglycin amino acid 101). This may be important for the final binding and fusion steps of fertilization and may serve to anchor and hold the 2 opposing gametes together, facilitating membrane fusion.

Mechanisms of action of ASA-positive seminal fluids in males and females following ejaculation have been well described (Bronson, 1999). In intact sperm, ESP lies within the acrosomal matrix (Wolkowicz et al, 2003). However, acrosome-reacted sperm may reveal exposed ESP in the cleft between inner and outer acrosomal membranes, thus providing sufficient antibody binding to cause the inhibitory effects observed if antibodies to ESP are present in oviductal fluids. Likewise, spontaneous acrosome reactions in both the male and female reproductive tracts may carry some intra-acrosmal ESP onto the plasma membrane. Anti-ESP antibodies present in seminal plasma may be predicted to affect fertility through this mechanism. Finally, the zona pellucida is known to be permeable to IgG. ASAs present in the zona and perivitelline space of sufficient titer may exert immunologic effects that are localized at the egg following the zona-induced acrosome reaction. Fine structural immunolocalization studies to date have not been successful in simultaneously preserving membranes and retaining immunogenicity of ESP sufficient to allow its precise localization with respect to the plasma membrane, inner and outer equatorial segment membranes, and matrix following the acrosome reaction and after egg binding.

ESP: Relationship to Other Equatorial Segment Proteins

Several proteins found to be associated with the equatorial region have been hypothesized to be involved in fertilization. One group thought to be involved in the final steps of fertilization is the ADAM family of proteins (Primakoff and Myles, 2000). This family, a subset of which is testis specific, contains both disintegrin and metalloprotease domains and therefore is potentially involved in both cell adhesion and protease activities. Indeed, antibodies to the testis-specific ADAM 2 (fertilin β) protein (Primakoff et al, 1987) and peptides representing the disintegrin domain of ADAM 2 (RGD motif; Evans et al, 1995) and ADAM 3 (cyritestin; Yuan et al, 1997) were found to block the adhesion and fusion steps of fertilization. More recently, knockout experiments with both ADAM 2 (Cho et al, 2000) and cyritestin (Nishimura et al, 2001) have demonstrated greatly reduced sperm-egg adhesion but no apparent involvement in the fusion step. ADAM 2 has been localized to the posterior of the head (Hunnicutt et al, 1997), not the equatorial segment.

The cysteine-rich secretory protein (CRISP) family of acidic proteins is thought to regulate calcium channels (Kirchoff, 1998), and some members are transcribed and secreted by the epididymis, where they associate with maturing spermatozoa. One family member, rat epididymal glycoprotein DE (named for isoforms “D” and “E”), associates with the dorsal sperm surface but migrates to the equatorial segment upon capacitation (Rochwerger and Cuasnicu, 1992). Antisera raised against recombinant forms of the mouse and human orthologs (Cohen et al, 2001) of this protein appear to inhibit sperm-egg fusion. However, unlike ESP's immunolocalization, the human ortholog reveals staining of the head as well as the principal and midpieces of the tail (Hayashi et al, 1996). Recently, targeted deletion of the intra-acrosomal protein IZUMO has been shown to result in complete infertility, with sperm entering the perivitelline space without difficulty but unable to fuse with the oolemma (Inoue et al,

2005). Antiserum to human IZUMO blocks human sperm fusion with the oolemma in the hamster egg model.

Previous investigations have provided evidence for subcompartments of the acrosomal matrix and membranes. During mouse spermiogenesis, the Golgi markers giantin, βCOP, golgin 97, and mannosidase II are localized to an acrosomal subcompartment that surrounds the region occupied by acrosin within the acrosomal granule (Ramalho-Santos et al, 2001). Similarly, the vSNARE, VAMP, and tSNARE syntaxin are present in a subdomain surrounding the acrosomal granule in mouse sperm (Ramalho-Santos et al, 2000) and are concentrated in human and rhesus sperm in the equatorial segment of non–acrosome-reacted sperm and persist on the equatorial segment following the acrosome reaction (Ramalho-Santos et al, 2000). Likewise, the cystatin-related CRES gene product has been shown to be present in the equatorial segment of human sperm (Wassler et al, 2002). Coimmunoprecipitation experiments are currently underway using the α–rec-hum-ESP serum to determine whether ESP is complexed or associated with any other equatorial segment proteins and to identify other constituents of the specific equatorial segment compartment that ESP occupies.

Useful ASA reagents have been generated to study sperm subcompartments (Bronson and Tung, 1992; Noor and Moore, 1999; Auer et al, 2000). Monoclonal antibodies HS1A.1 (Villaroya and Scholler, 1986) and MA1-3 (Isahakia and Alexander, 1984) recognize proteins residing only in the principal segment of the human acrosome, whereas D3 (Hinrichsen-Kohane et al, 1985) and 21D3 (Le et al, 1984) bind antigens located only in the equatorial segment. Olson et al (1998) have demonstrated immunohistochemically that 2 major acrosomal matrix proteins, AM22 and AM29, are excluded from the equatorial segment of mouse sperm, whereas the MN9 monoclonal antibody (Toshimori et al, 1992) recognizes 38- and 48-kDa proteins in mouse that are segregated specifically to the equatorial segment. Other researchers reported that the M1 (Noor and Moore, 1999) and P36 (Auer et al, 2000) monoclonal antibodies, which recognize sperm protein bands of 38 and 34 kDa in hamster and humans, respectively, localize to the equatorial segment and inhibit fertilization. However, while the identities and functions of the proteins corresponding to these immunoreagents remain unknown, there is a striking similarity in molecular weight to isoforms of ESP.

Clinical Implications for Infertility

ASAs have been identified in 10%–15% of men experiencing infertility and 15%–20% of women with unexplained infertility (Ghazeeri and Kutteh, 2001). ASAs persist for years following vasectomy and are thought to be one reason for the infertility that may follow vasectomy reversal (for reviews, see Bronson et al, 1994; Marshburn and Kutteh, 1994; Bronson, 1999). The presence of ASAs in clinical cases of infertility, therefore, indicates a possible etiology for the inability to conceive on the part of either the male or female partner. The present experiments demonstrate that ASA-positive infertile male and, to a lesser degree, infertile female sera reacted positively with the 36- to 38-kDa ESP region on 2D gels of human sperm protein extracts, indicating that this antigen is both accessible to and immunogenic for the human immune system. Our live staining, immunofluorescence, and electron microscopy observations (Wolkowicz et al, 2003) have shown ESP to be present within the acrosome, bringing into question how the ESP protein could elicit an immune response in males. It will be noted that some sperm undergo a spontaneous acrosome reaction, thus releasing their acrosomal contents prematurely. Indeed, exposure of the male immune system to the acrosomal contents in the urethra or elsewhere in the postepididymal male reproductive tract may cause the production of antibodies and contribute in some cases to an antifertility effect in the male. Likewise, this same mechanism of premature, spontaneous acrosome reaction could elicit antibodies in the female's lower reproductive tract and infertility via vaginal secretion of antibodies. Endogenously generated ASA-positive sera recognized both native and

recombinant ESP on Western blots, indicating the rec-h-ESP retains sufficient primary and secondary structure to be recognized by antibodies to the native antigen. Polyclonal immune sera raised against the cloned recombinant ESP showed that endogenous ESP was localized to the equatorial segment, where it was retained in acrosome-reacted sperm. This same anti-ESP antiserum significantly blocked sperm binding and, more importantly, fusion when tested in vitro. This result is in accord with the finding that ESP is retained in the acrosomes of all sperm tightly bound to the oolemma.

Taken together, these data suggest ESP is an antigen that may be involved in immune infertility in humans and that recombinant ESP may be useful to monitor infertility-associated antibody responses. Intracytoplasmic sperm injection (ICSI) is now the standard of care in many cases of ASA as well as other forms of male infertility. Presently, the incidence of transmission of infertilities of genetic origins (such as motility defects) to the male offspring after ICSI is poorly understood, because the first ICSI males are only now reaching puberty. Interest in differential diagnosis of the causes of male immunoinfertility, such as ASA, may become more important in the future if the acceptability of ICSI in treating some forms of infertility were to wane due to a high rate of transmission of infertilities of genetic origin. In the future, ICSI might continue to be the management tool of choice in the case of ASA, whereas it might not be the management tool of choice for certain infertilities with transmissible genetic origins. A differential diagnosis of immunoinfertile patients with ESP and other molecules would be helpful in deciding which patients should be treated with ICSI therapy. Moreover, ESP's testis-abundant expression, presence on acrosome-reacted sperm and on sperm bound to eggs, as well as the inhibition of fertilization by antibodies to recombinant ESP, indicate that ESP may also be a candidate contraceptive vaccinogen. Currently, we have a number of sperm proteins, including ESP, undergoing testing in macaques to ascertain their immunogenic properties (Herr, 1996; Diekman and Herr, 1997; Kurth et al, 2007).

In addition to previous strategies for establishing the identities of the target antigens recognized by ASAs (Herr et al, 1985; Tsuji et al, 1988; Diekman et al, 1999; Li et al, 2000), the present report shows that screening of 2D Western blots with ASA-positive sera and mass spectrometry can result in the characterization of relevant antigens with key roles in fertilization and demonstrates that proteomic strategies are a powerful approach to dissect the molecular mediators of immunoinfertility.

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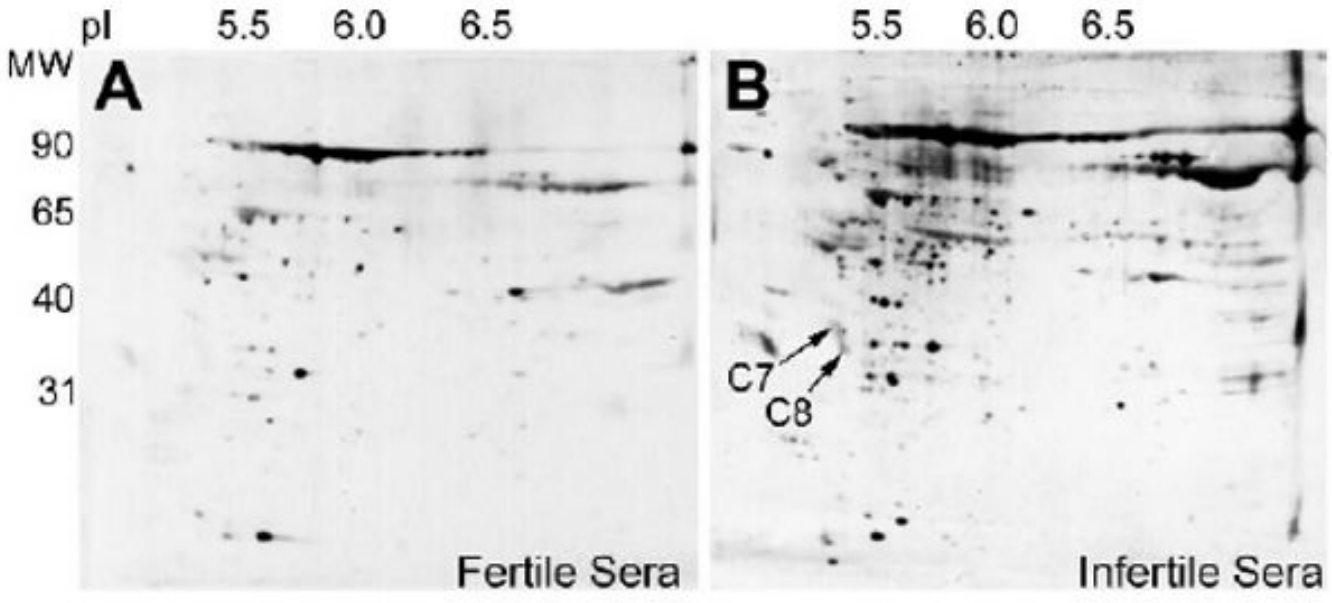

Figure 1.

Two-dimensional (2D) (pI 4.8–7.0) immunoblot comparison of NP-40/urea-solubilized human sperm proteins immunoreactive with fertile vs infertile ASA-positive human sera. Molecular weight standards ($\times 10^{-3}$) are indicated in the left margin, and isoelectric points are indicated at the top. **(A)** A pool of sera from 5 fertile individuals incubated with the 2D blot revealed several high– and low–molecular weight immunoreactive human sperm proteins. **(B)** The pooled sera of 5 ASA-positive infertile male patients detected a much larger number of immunoreactive sperm proteins, among them spots C7 and C8 (arrows), which when cored yielded ESP microsequences. Goat α-human secondary antibody binding was visualized by enhanced chemiluminescence. Parallel experiments with a pool of sera of 5 fertile individuals vs 5 ASA-positive infertile female patients recognized a slightly different array of sperm antigens and also included ESP (data not shown).

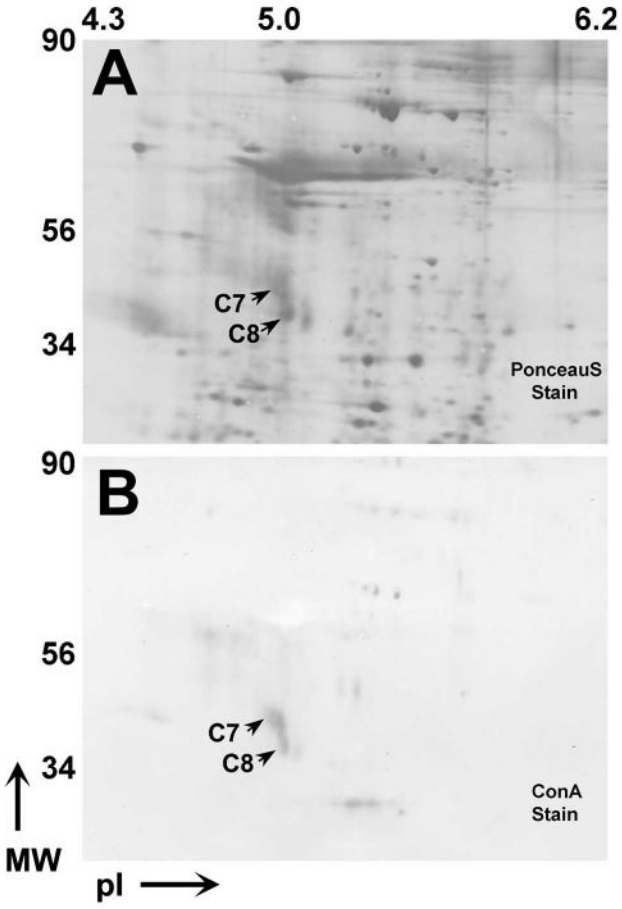

Figure 2.

C7 and C8 spots react with both Con-A and antibodies specific to ESP. Spots C7 and C8 immunoreactive with ASA-positive patient sera (Figure 1) stained on high-resolution 2D Western blots of human sperm proteins with **(A)** Ponceau S and **(B)** Con-A. On this high-resolution 2D immunoblot, multiple charge variants of ESP were noted, as well as a few lower–molecular weight degradation products. In conjunction with the recovery of ESP microsequences, this immunologic proof verified ESP as a sperm alloantigen. Antibodies to rec-h-ESP also reacted to this specific area (see Figure 4, panel 3B in Wolkowicz et al, 2003, for comparison).

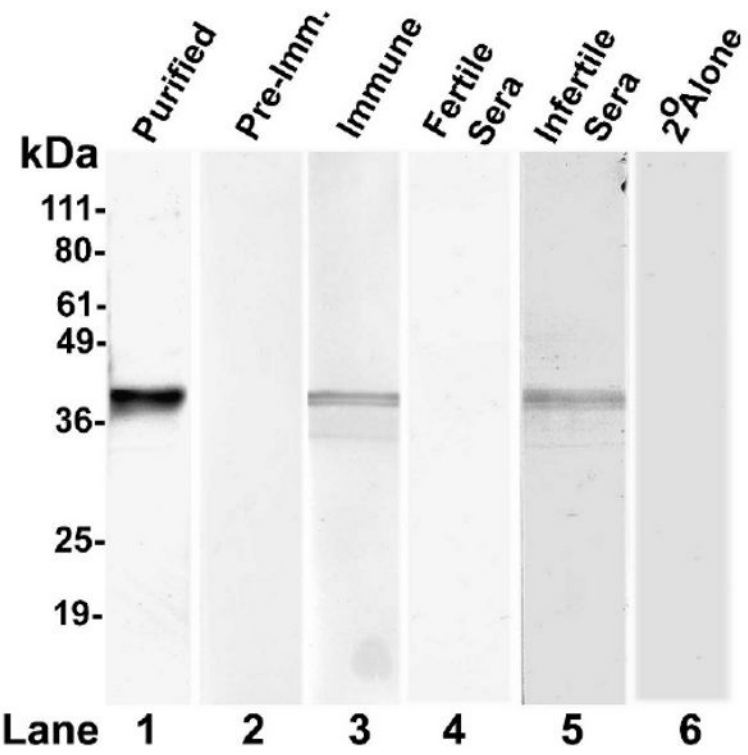

Figure 3.

ASA-positive human sera react with recombinant ESP. Lane 1, Coomassie-stained gel of purified recombinant ESP protein (38 kDa). Lanes 2 through 6, 12.5% polyacrylamide gel electrophoresis immunoblots of rec-h-ESP (0.1 μg) reacted with preimmune (lane 2) and immune (lane 3, 1:16 000) rat sera generated to rec-h-ESP or human fertile (lane 4, 1:1000) or infertile ASA-positive sera (lane 5, 1:1000). Goat anti-rat (1:5000, lanes 2 and 3) or goat anti-human secondary antibodies (1:5000, lanes 4, 5, and 6) and diaminobenzidine, respectively.

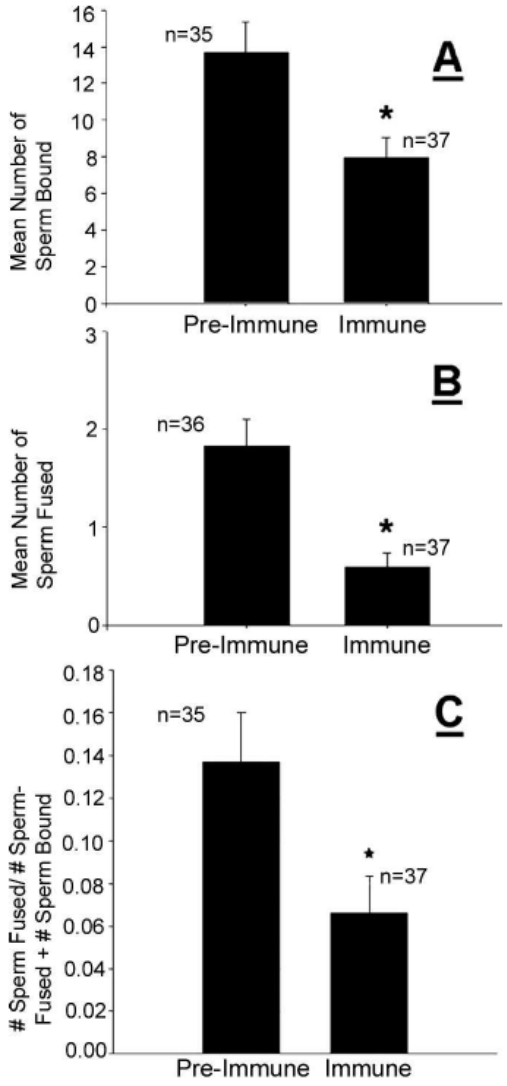

Figure 4.

Effects of α-rec-ESP antibodies on the hamster egg penetration xeno-assay. **(A)** Mean number of spermatozoa bound per oocyte in the presence of 1:50 dilutions of preimmune and immune rat α-rec-ESP sera. Binding was scored using phase contrast microscopy. **(B)** Sperm-egg fusion scored by counting the number of swollen heads within each acridine orange–stained oocyte using fluorescence microscopy. **(C)** Differences between preimmune and immune sera expressed as fused sperm/bound + fused sperm. Bars represent ± SD of 3 individual experiments. n = total number of oocytes per group. * indicates $P \leq .05$ (Student's *t* test was used throughout to derive all *P* values).

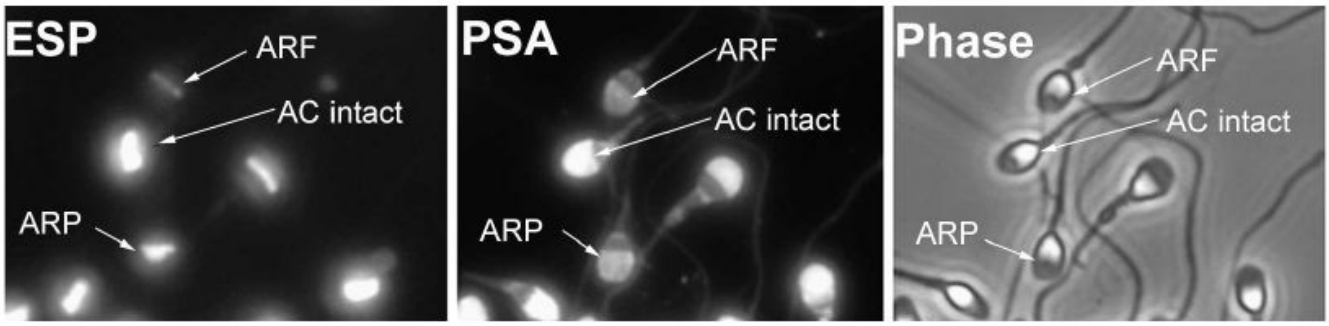

Figure 5.
Dual staining of human sperm with both anti-ESP antibodies and PSA. Anti-ESP antibodies stain the equatorial region, whereas the same field stained with PSA revealed acrosome-intact (AC intact), partial (ARP), and fully acrosome-reacted (ARF) sperm. Right panel shows the phase contrast photo of same field, magnification × 975.

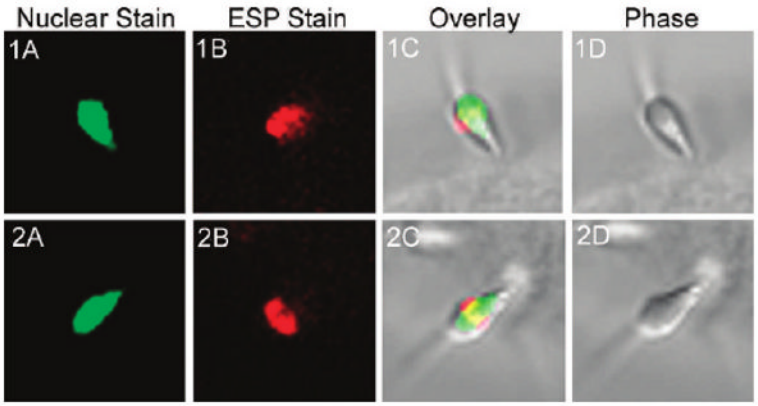

Figure 6.

Immunofluorescent localization of the ESP protein on sperm tightly bound to hamster eggs after the hamster egg penetration assay. Capacitated human sperm were incubated with zona-free hamster eggs and washed, fixed, and dual stained with rat α-rec-ESP sera and Sytox. Phase contrast images (**1D** and **2D**) of 2 of 6 sperm bound to this egg are compared to the same sperm visualized for nuclear chromatin staining with Sytox (green fluorescence, **1A** and **2A**) and with immune α-rec-ESP sera visualized with FITC-labeled secondary antibody (red fluorescence, **1B** and **2B**). **Panels 1C** and **2C** represent the overlay of chromatin and ESP domains in oolemmal-bound sperm. ESP location in the equatorial segment is demarked by yellow areas where red and green stains overlap. The other 4 sperm on this egg visualized by confocal microscopy also were ESP positive. Of 78 sperm observed on 10 eggs, 100% were ESP positive, indicating that ESP is retained after tight binding of human sperm to hamster eggs. Magnification × 1566.

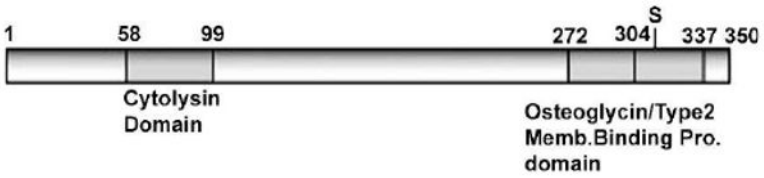

Figure 7.

ESP sequence and homology domains. Figure to scale. Numbering above designates the amino acids at the beginning and end of each domain. In the osteoglycin domain, the location of the serine residue responsible for chondroitin sulfate binding is noted (S). Cytolysin domain, aa58–99; Type 2 membrane-binding protein domain, aa274–304; Osteoglycin domain, aa272–337.

Table

Retention of ESP in acrosome-reacted sperm[a]

Treatment	+ESP/−AcR	+ESP/+AcR	−ESP/−AcR	−ESP/+AcR	Total	% +AcR	% +ESP	% AcR Also +ESP
Noncapacitated	537 (91.8)	31 (5.3)	15 (2.6)	2 (0.3)	585	5.6	97.1	93
Capacitated	464 (85.1)	68 (12.5)	8 (1.5)	5 (1.0)	545	13.4	97.6	93
EtOH	456 (83.4)	70 (12.8)	12 (2.2)	9 (1.6)	547	14.4	96.2	89
Progesterone	438 (79.5)	87 (15.8)	15 (2.7)	11 (2.0)	551	17.8	95.3	89

[a]Presence of ESP in acrosome-reacted sperm. Fresh swim-up sperm from 5 individuals were dually stained with PSA-lectin and α–rec-ESP antibody. The acrosome reaction was assessed by loss of PSA lectin staining from the principal segment of the acrosome. Samples were scored as −AcR, indicating full PSA-positive acrosomal cap (principal segment), or +AcR, indicating diminution or absence of a PSA-positive principal segment. Samples consisted of noncapacitated swim-up sperm, capacitated sperm treated with ethanol vehicle alone, and capacitated sperm treated with 5 μg/mL progesterone. ESP was retained in 89% of acrosome-reacted sperm, identified by a decrease or absence of PSA staining. The numbers in the parentheses indicate percentage of total sperm observed in the category.

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