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Identification of human sperm proteins involved in the interaction with homologous zona pellucida

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Objective: To identify human sperm proteins involved in homologous zona pellucida (ZP) interaction.

Design: Prospective study.

Setting: Basic research laboratory.

Patient(s): Semen samples from normozoospermic donors, tissue sections from surgical pieces, and ZP from nonfertilized oocytes.

Intervention(s): Antibodies for sperm proteins (HSE; high salt extract) were developed (anti-HSE) and partially characterized. Participation of sperm proteins on ZP-interaction was tested with the hemizona assay (HZA). Antigens were immunolocalized in sperm and tissues.

Main Outcome Measure(s): Sperm and tissue immunostaining; Western blotting; and number of sperm bound to the ZP.

Result(s): Anti-HSE antibodies recognized several polypeptides in HSE (9 to 200 kd). Specific antibodies for 49 and 66 kd proteins (p49, p66) were obtained. Both (anti-p49 and anti-p66) stained the head of ejaculated and capacitated sperm. In the HZA, sperm preincubation with a mixture of anti-p49 and anti-p66 (100 μg/mL) resulted in a decrease in the number of spermatozoa bound to the ZP. Presence of p66 (10 μg/mL) inhibited sperm–ZP interaction. In contrast, p49 did not alter sperm binding to the ZP. Immunohistochemical analysis showed that p66 is present in the epididymis. No staining was observed in testicular sections.

Conclusion(s): We found that p66 is an epididymal protein that participates in human sperm interaction with homologous ZP. (Fertil Steril® 2003;79(Suppl 3):1606–15. ©2003 by American Society for Reproductive Medicine.)

Key Words: Human, spermatozoa, zona pellucida, antibodies, epididymis, sperm-egg interaction

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Fertilization results from successful interaction between the female and male gametes. Capacitated human spermatozoa initially bind, in a species-specific manner, with the zona pellucida (ZP), an extracellular coat that surrounds all mammalian eggs. As a result of this interaction, spermatozoa undergo an exocytotic process called the acrosome reaction, which allows sperm penetration through the ZP and fusion with the oocyte plasma membrane (1, 2). In many mammals, including humans, it has been shown that the ZP is composed of three glycoproteins, called ZP1, ZP2, and ZP3, which are well characterized at biochemical and molecular level (3, 4). Of the three components, ZP3 is responsible for primary sperm binding to the ZP and induction of the acrosome reaction (AR) (5, 6), while ZP2 would participate in secondary binding to the ZP (7).

In contrast with the ample information available about ZP composition and role during fertilization, the molecular identity and biochemical characterization of protein sperm receptor(s) involved in binding to the ZP have yet to be completely elucidated. In humans, several proteins have been proposed as participating in primary and secondary binding to the ZP. Within those are the FA-1 antigen (8), the zona receptor kinase (ZRK) (9), P34H (10), a mannose-lectin (11), β-hexosaminidase (Hex, E.C.3.2.1.52) (12), an antigen recognized by the monoclonal antibody 4A8 (13), selectins

(14), YLP12-protein (15), anti-SLIP reactive proteins (16), SOB3 (17), PH20 (18, 19), SP10 (20), and proacrosin (E.C.3.4.21.10) (21). The sequence encoding some of these proteins has been reported (9, 22–25). However, none of them have proven to be essential in sperm–egg interaction.

Considering the relevance of the initial steps in the interaction between spermatozoa and the ZP, identifying the sperm proteins involved in that process is of great importance to understanding the mechanism of fertilization. Moreover, such an understanding may contribute to the development of new diagnostic assays, and eventually may help in the treatment of male infertility.

In the present study, we performed a set of experiments to identify human sperm proteins of epididymal origin involved in ZP interaction. The strategy selected involved preparation of sperm extracts of surface proteins that were used as antigen in the development of polyclonal antibodies. Specific antibodies for selected proteins were used to assess protein localization in spermatozoa and tissue sections from the male tract. In addition, these antibodies and the isolated proteins were tested for their ability to block sperm–ZP interaction in the hemizona assay (HZA).

MATERIALS AND METHODS

Materials

Semen samples were obtained from normozoospermic donors according to World Health Organization standards (26). Only samples with more than 90% live spermatozoa and 75% progressive motile cells were included in the study. Sperm vitality was assessed using 0.5% eosin Y in 0.9% sodium chloride. Seminal plasma (SP) was obtained after semen centrifugation at 10,000 $\times$ *g* for 10 minutes.

Human epididymal and testicular tissues were obtained from patients undergoing orchiectomy as treatment for prostatic carcinoma, who had not received any hormonal treatment before surgery. Testicular and epididymal protein extracts were obtained after tissue homogenization in 4 volumes of phosphate buffer saline (PBS) per tissue gram, followed by centrifugation at 105,000 $\times$ *g* for 1 hour at 4°C (cytosol fraction). Cauda epididymal plasma (CEP) was obtained by retrograde perfusion of the vas deferens, as previously described (27).

Unless specified, all reagents were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO), Bio-Rad (Hercules, CA), and Life Technologies Invitrogen (Gaithersburg, MD).

This research study was approved by the review board of the Argentine Society of Clinical Investigation. Tissue, semen, and fluid samples were obtained with the informed written consent of the patient or donor.

Methods

Electrophoresis and Immunoblotting

We performed SDS-PAGE in 8% or 10% polyacrylamide gels with 0.1% SDS (28). Samples under analysis were prepared in Laemmli sample buffer in the presence of 5% 2-β-mercaptoethanol, and boiled for 5 minutes. Prestained molecular weight markers (Life Technologies) were run in parallel in all cases.

Proteins were visualized by staining with Coomassie R-250 brilliant blue stain (CBB). For Western immunoblot analysis, proteins were electroblotted as previously described (29) onto nitrocellulose membranes, and then were immunostained with different antibodies. Membranes were placed in blocking solution (5% skim milk in phosphate buffer saline, PBS, containing 0.1% Tween 20) for 1 hour, washed using PBS with 0.1% Tween 20, and incubated overnight with the first antibody (anti-HSE or anti-p66 and anti-p49 antibodies) in blocking solution at 4°C. Nitrocellulose membranes were washed, incubated for an additional hour with anti-rabbit IgG coupled to peroxidase in blocking solution, and developed with enhanced chemiluminescence (ECL kit, Amersham Life Science, Buckinghamshire, UK). Blot immunostaining was performed at room temperature with constant shaking.

Immunostaining of Spermatozoa and Tissues

Human spermatozoa from normozoospermic donors were fixed in 2% formaldehyde in PBS for 4 minutes loaded in polylysine-coated microscope slides, allowed to dry at room temperature, and incubated overnight with the first antibody (anti-HSE, anti-p66, anti-p49; 1:100 dilution) at 4°C in a humid chamber. Following three washes of 5 minutes in PBS, anti-rabbit IgG-FITC in PBS supplemented with 4% bovine serum albumin (BSA) was added and incubated for 1 hour at room temperature in the darkness.

After three washes, cells were mounted and observed in a Nikon fluorescence microscope coupled to an image analyzer (IPLab Scientific Imaging Software for Windows; Scanalytics, Inc., Fairfax, VA). The immunostaining procedure was performed on ejaculated spermatozoa devoid of seminal plasma, and on spermatozoa incubated in vitro for 4 hours under capacitating conditions with human sperm medium (HSM) (30) containing 2.6% BSA (4-hour capacitated spermatozoa). Before fixation and staining procedures, cells were extensively washed to remove remnant BSA from the medium.

For immunohistochemical evaluations, small portions of human testis and epididymis (caput, corpus, cauda) were fixed for 24 hours at 4°C in methanol/acetic acid (95:5) and embedded in paraffin. Tissue sections of 4 μm were sequentially incubated with 2% H_2O_2 in PBS to block endogenous peroxidase, with 4% BSA in PBS, and with 15% normal rabbit serum in PBS to suppress nonspecific binding. Pri-

mary antibodies (1:50) were applied overnight at room temperature in PBS containing 1% BSA (blocking solution), followed by several washes with PBS containing 0.02% Tween 20 to remove the unbound antibody, and incubation with a 1:2,000 dilution of biotinylated goat anti-rabbit IgG in blocking solution at room temperature. Finally, samples were incubated with avidin/peroxidase (1:200), and developed with 1 mg/mL 3,3′-diaminobenzidine tetrachloride in PBS containing 0.3% H_2O_2. Sections were analyzed at 400× and 1,000× magnification.

Assessment of the Acrosomal Status

The acrosomal status of ejaculated spermatozoa, as well as of cells incubated for 4 hours in a capacitating medium, was assessed. In all cases, at the end of the incubation period, cells were extensively washed, fixed, and stained with the lectin *Pisum sativum* agglutinin (Sigma) labeled fluorescein isothiocyanate (FITC), as previously described (31). Spermatozoa were analyzed at 1,000× magnification. The presence of bright staining over the acrosomal cap was indicative of an intact spermatozoa; cells lacking stain in the acrosome were scored as acrosome-reacted.

Preparation of Protein Extracts and Polyclonal Antibodies for Human Sperm Surface Proteins

Over 4,000 million spermatozoa obtained from 10 semen donors and freed of seminal plasma components were separated from the SP by centrifugation, then were resuspended and incubated for 20 minutes in 0.1 M pipes buffer (pH 7.4) supplemented with 1 M NaCl, 0.25 M sucrose, and a cocktail of proteinase inhibitors. Sperm suspensions were subjected to centrifugation at 15,000 × *g* for 10 minutes, and the supernatant was recovered, dialized against distilled water, and resuspended in 50 mM tris-HCl in the presence of proteinase inhibitors. The protein extract obtained was named HSE (high salt extract).

Anti-HSE polyclonal antisera were developed in four New Zealand rabbits. In each case, aliquots of 200 μg of HSE were injected SC with complete Freund adjuvant, followed by two injections with incomplete adjuvant every 21 days. Once the immunization protocol was completed, sera was collected and analyzed by Western immunoblotting of HSE proteins with a 1:1,000 dilution of the immune serum, followed by incubation with peroxidase conjugated goat anti-rabbit IgG (1:1,000 in blocking solution), and development with enhanced chemiluminescence. Animal handling was conducted in accordance with standard guiding principles for the care and use of research animals.

Preparation of Antibodies for Specific Sperm Proteins

Anti-HSE immune sera were adsorbed for two major HSE protein regions of 66 kd (p66) and 49 kd (p49). We isolated p66 and p49 proteins after 8% preparative SDS-PAGE followed by Western immunoblotting to nitrocellulose membranes, located them by staining with Ponceau solution (0.2% *w*/*v* in 0.5% of acetic acid in water), and estimated their Mr. To confirm localization of the immunoreactive bands, both membrane edges were subjected to the immunostaining protocol with anti-HSE and aligned to the remnant membrane. The anti-HSE immune serum was incubated (1:50) for 18 hours with each protein band, and washed to remove excess of unbound antibody. Immunoglobulins were desorbed from the nitrocellulose membranes with a 0.2 M glycine solution (pH 2.5), and neutralized with tris-HCl 0.1 M (pH 8.0).

The immunoglobulin fractions were tested in immunocytochemistry of spermatozoa, as well as in Western immunoblotting of spermatozoa and male reproductive tissue protein extracts. In addition, these antibodies were used in the HZA, and in immunohistochemistry of epididymal and testicular tissue sections.

Preparation of p66 and p49 Proteins

We subjected HSE proteins to 8% SDS-PAGE, allowing an effective separation of p66 and p49 protein bands. Gel pieces of each band were recovered and placed in 10 mM ammonium acetate (pH 7.0) for 24 hours for passive protein elution. Eluates of p66 and p49 protein bands were concentrated by centrifugation (Centricon 30, Amicon, Millipore; Bedford, MA), and the protein content was assessed by Bradford (BioRad) following a protocol suggested by the manufacturers. Purity of the isolated proteins in the eluates was evaluated by SDS-PAGE, followed by silver staining, showing in both cases only one band of the expected Mr. Both eluates were used in the HZA.

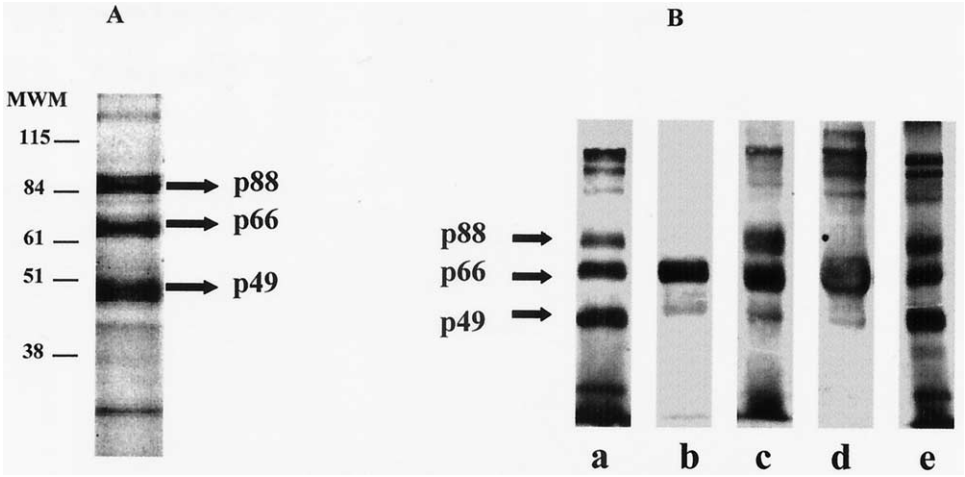
Hemizona Assay

The HZA was done as previously described (32). Incubations were carried out in HSM supplemented with 3.5% BSA, and in which 2.5 mM $CaCl_2$ had been replaced by the same amount of $SrCl_2$. This condition has been previously shown to support sperm capacitation–related events and sperm interaction with homologous ZP, but does not support the induction of the acrosome reaction that may occur in cells incubated in the presence of certain antisperm antibodies (33). Each hemizona was placed in a 100-μL drop of a suspension containing 6×10^4 motile spermatozoa which had been previously incubated in the capacitating medium for 4 hours, followed by a 1-hour incubation with 100 μg/mL of a mixture of anti-p66 + anti-p49 antibodies or normal rabbit serum (control condition).

In a second set of experiments, the purified proteins (p66, p49) under evaluation were added to the incubation medium. After a 4-hour incubation period, the hemizonae were washed by repeated vigorous pipetting in drops of medium, and the number of spermatozoa tightly bound to the outer surface was counted under a 400× magnification using Hoffman interference optics (Modulation Optics Inc., Greenvale, NY).

FIGURE 1

HSE proteins and immunoreactivity of anti-HSE. (**A**) High salt extract (HSE) components were separated in 8% SDS-PAGE and stained with CBB. Samples were reduced by adding 5% 2-β-mercaptoethanol before electrophoresis. MWM: molecular weight markers (kd). This experiment was performed at least five times obtaining similar results. A typical experiment is shown. (**B**) Protein extracts were subjected to 8% SDS-PAGE, followed by Western immunoblotting with anti-HSE. This experiment was Performed at least five times obtaining similar results. A typical experiment is shown. (*Lane a*) High salt extract (HSE) proteins. (*Lane b*) Proteins from human epididymis. (*Lane c*) Cauda epididymal plasma (CEP) proteins. (*Lane d*) Proteins from human testis. (*Lane e*) Seminal plasma proteins.



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Statistical Analysis

Data from the HZA were expressed as mean ± standard deviation (SD). The number of spermatozoa bound to HZ subjected to different experimental conditions was compared using the Wilcoxon test. Statistical analyses were done with an IBM compatible computer using the GraphPad InStat program (GraphPad Software, San Diego, CA).

RESULTS

Development and Partial Characterization of Anti-HSE Antibodies

Sperm treatment with a buffer containing 1 M NaCl resulted in the extraction of a complex mixture of proteins, as seen in the pattern obtained after SDS-PAGE analysis followed by CBB protein staining (Fig. 1A). An incubation period of 20 minutes in this solution was sufficient to effectively extract an abundant amount of proteins of an ample molecular weight range (9 to 200 kd). Sperm treatment under these experimental conditions appeared to mainly extract surface polypeptides without significantly disrupting the plasma membrane integrity, as suggested by the high percentage of live cells (higher than 75%) recovered after incubation with the protein extraction buffer.

The HSE was used to immunize four rabbits. In all cases, sera from the injected animals gave similar results (data not shown). Based on that, a pool of the four sera was made and used for all evaluations. In Western blots of HSE proteins, immunoglobulins from the anti-HSE immune serum recognized several sperm components, showing a strong signal for abundant proteins of 88, 66, and 49 kd (p88, p66, and p49, respectively) (Fig. 1B, lane a). Anti-HSE antibodies recognized several testicular and epididymal proteins in Western blots of protein homogenates from both tissues (see Fig. 1B, lanes b and c, respectively). In addition, a positive reaction was obtained for polypeptides from CEP and SP (see Fig. 1B, lanes d and e, respectively).

By immunocytochemical analysis, ejaculated spermatozoa incubated with anti-HSE antibodies showed a specific staining mainly over the acrosomal cap and tail in 70% of the cells; 28% also showed a weak signal in the midpiece and tail (Fig. 2a). In capacitated cells, the staining was intense and mainly associated to the acrosomal region in 85% of the cells (see Fig. 2b). Immunohistochemical analysis of tissue sections from human testis and epididymis revealed a specific positive signal in both cases (data not shown).

Antibodies for Proteins p66 and p49: Preparation and Characterization

Two abundant HSE proteins, named p66 and p49 after their Mr, were subjected to further analysis. Specific antibodies for both polypeptides were obtained by incubation of anti-HSE antibodies with the immobilized proteins as described in the Material and Methods section.

FIGURE 2

Immunolocalization of p49 and p66 in spermatozoa. Immunocytochemical analysis of ejaculated (a, c, e) and 4-hour capacitated (b, d, f) spermatozoa stained with anti-HSE (a, b), anti-p66 (c, d), and anti-p49 (e, f) antibodies. Staining performed with preimmune serum (g: bright-field, h: immunofluorescence) (magnification, ×400).

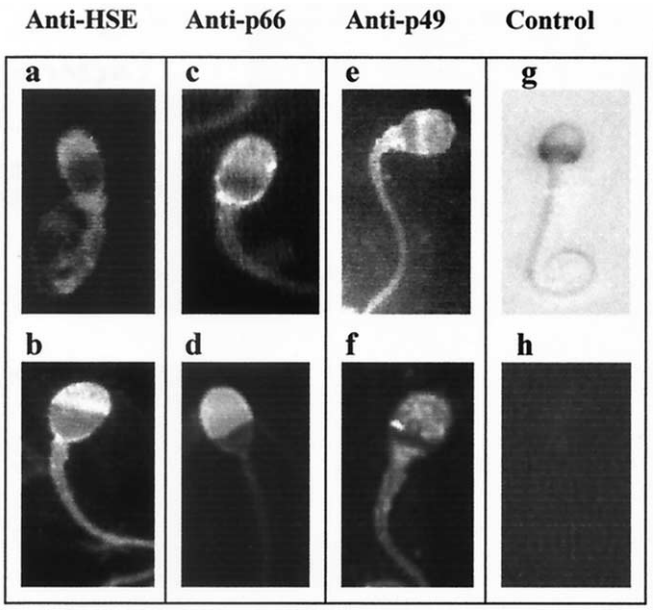

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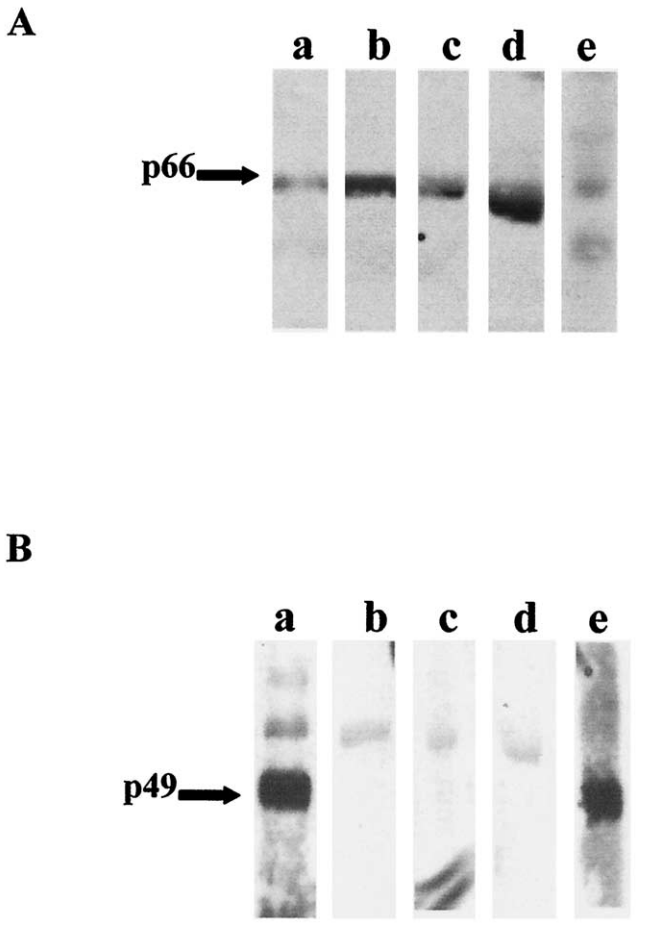
FIGURE 3

Immunoreactivity of anti-p49 and anti-p66 antibodies for proteins from the male reproductive tract. Protein extracts from the male reproductive tract were subjected to 8% SDS-PAGE followed by Western immunoblotting with anti-p66 (**A**) and anti-p49 (**B**) antibodies. (*Lane a*) High salt extract (HSE) proteins. (*Lane b*) Proteins from human epididymis. (*Lane c*) Cauda epididymal plasma (CEP) proteins. (*Lane d*) Proteins from human testis. (*Lane e*) Seminal plasma proteins.



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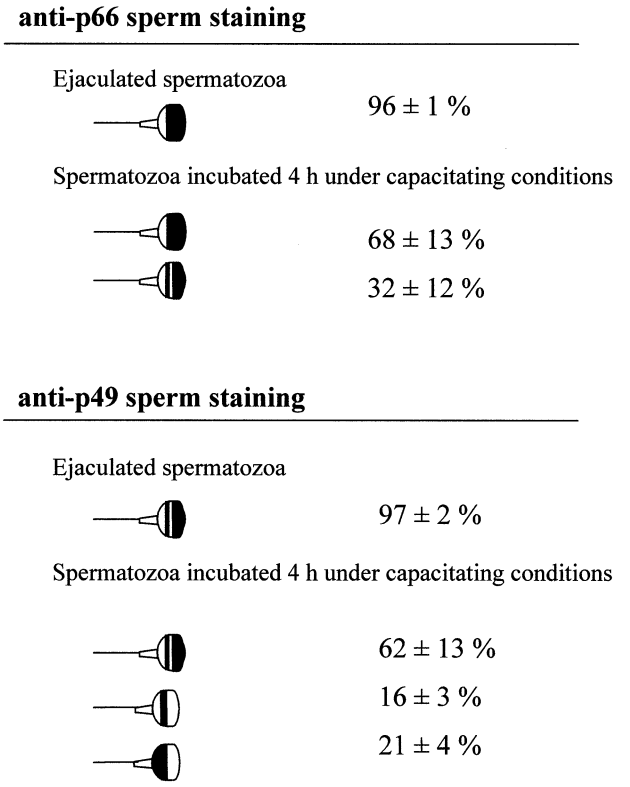
Western immunoblot analysis of HSE proteins with anti-p66 antibodies gave a positive signal for the antigen (p66), though a weak cross reactivity was observed in the 49 kd region (Fig. 3A, lane a). In protein extracts from the male reproductive tract, anti-p66 antibodies showed a strong signal with a 66 kd band in epididymal cytosol (lane b) and detected a protein with a similar Mr in CEP (lane c). In testis, a protein was also recognized, though the Mr was slightly smaller (lane d). Finally, at least two polypeptides of 66 and 49 kd gave a weak signal with anti-p66 in seminal plasma (lane e). A similar analysis done using anti-p49 antibodies revealed a strong signal with a 49-kd protein only in HSE and seminal plasma; in addition, a weak cross reactivity for p66 was detected in all protein extracts but SP (see Fig. 3B, lanes a and e, respectively).

Localization of p49 and p66 antigens in spermatozoa was investigated by immunofluorescence analysis of ejaculated and capacitated cells. In ejaculated sperm, anti-p66 epitopes were detected on the acrosomal cap in over 95% of the cells (see Fig. 2c; Fig. 4). After incubation in a capacitating medium for 4 hours, p66 remained mainly localized to the sperm acrosome (see Fig. 2d; Fig. 4), though around 30% of the cells showed an additional signal in the equatorial ring (see Fig. 4).

When the staining procedure was done with anti-p49 antibodies, 97% of ejaculated spermatozoa showed a specific signal in the equatorial ring and the neck, and a patchy pattern over the acrosomal cap (see Fig. 2e; Fig. 4). Over 80% of 4-hour capacitated sperm showed a strong staining in the neck, which was found alone or in combination with a patchy signal in the acrosomal cap (see Fig. 2f; Fig. 4). Staining in the equatorial segment remained in only 16% of the cells. Staining with both antibodies was specific, because omission of the first antibodies resulted in a lack of signal (see Fig. 2h). Low percentages of spontaneous acrosome-reacted cells were found in both conditions (ejaculated: 9%

FIGURE 4

Immunolocalization of p66 and p49 in ejaculated and 4-hour capacitated spermatozoa. Assessment of sperm staining patterns with anti-p66 and anti-p49 antibodies was done in at least 200 spermatozoa from four semen samples.



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± 1%, 4-hour capacitated: 7% ± 2% acrosome-reacted spermatozoa; n = 3).

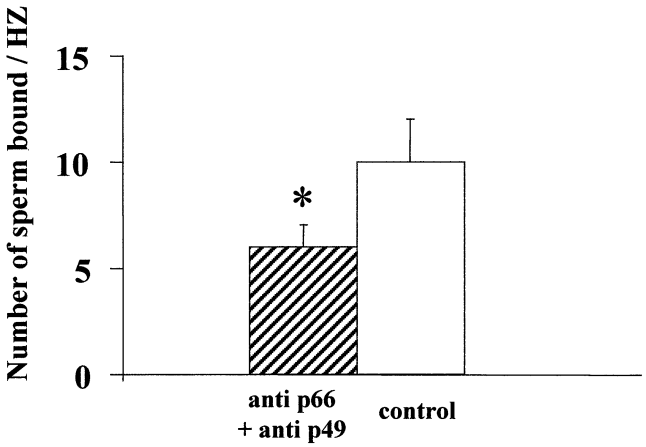
Participation of p66 and p49 Sperm Proteins in the Interaction With Homologous ZP

To test whether p66 and/or p49 participate in sperm–oocyte interaction, HZA was done using antibodies for these proteins, and the proteins themselves. In a first set of studies, the effect of sperm preincubation with a mixture of anti-p66 and anti-p49 antibodies upon the interaction with the ZP was evaluated. As shown in Figure 5, a significant decrease in the number of spermatozoa bound to the ZP was observed when cells were preincubated with the antibodies (anti-p66 + anti-p49: 6 ± 2, vs. control: 10 ± 3, $P<.01$; n = 6), representing a 47% inhibition of the binding. The percentage of motile cells at the end of the incubation period was similar to that before the addition of the immunoglobulins (range 70% to 85% motile cells).

Proteins p66 and/or p49 were recovered from preparative gels and added to the gamete incubation drop at 10 μg/mL

FIGURE 5

Effect of anti-p66 and anti-p49 antibodies on sperm–ZP interaction. Spermatozoa were preincubated with 100 μg/mL of a mixture of anti-p49 + anti-p66 antibodies or preimmune serum (control) and washed, followed by incubation with homologous ZP using the HZA. In all cases, spermatozoa tightly bound to the outer face of each HZ were counted. Results show the number of spermatozoa bound to each HZ (n = 6) (mean ± SD).



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final concentration to test whether they exert an inhibitory effect on sperm–ZP interaction. Presence of p66 during incubation of spermatozoa with the ZP resulted in a significant decrease in the number of cells bound/HZ with respect to controls (9 ± 2 vs. control: 19 ± 4, $P<.01$; n = 5; Fig. 6A), representing a 60% inhibition of ZP binding.

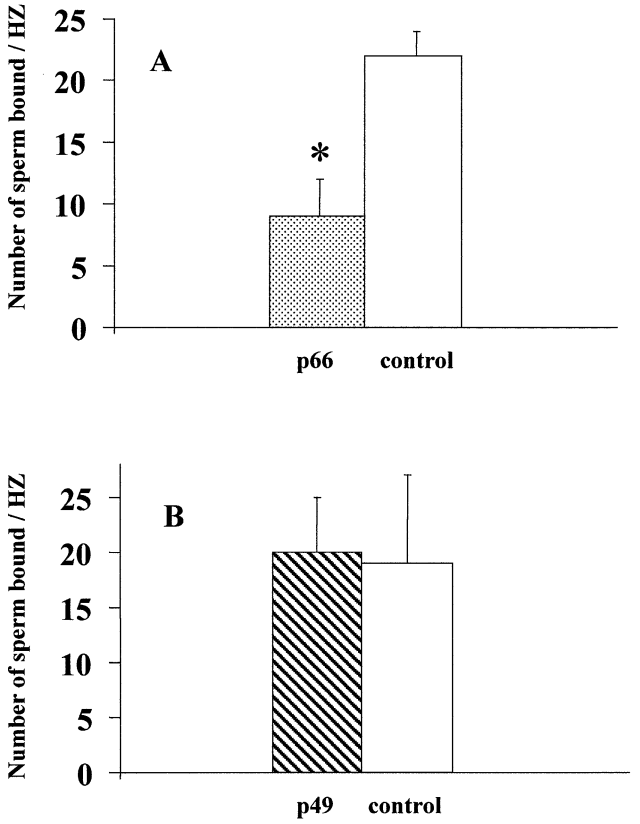
In contrast with these findings, gamete incubation in the presence of p49 did not alter sperm interaction with the ZP. A similar number of spermatozoa bound in each HZ was found when incubations were carried out in the presence or absence (control) of 10 μg/mL of p49 (20 ± 4, control: 19 ± 7, NS; n = 5; see Fig. 6B). Because contamination of the p66 fraction with human serum albumin (HSA) was occasionally detected by Western immunoblotting (data not shown), the HZA was done in the presence of HSA (10 μg/mL). Similar numbers of spermatozoa bound to the HZA in the presence or absence of HSA (20 ± 6 vs. control: 18 ± 6; n = 7).

Tissue Localization of p66

To evaluate tissue localization of p66 in the male reproductive tract, we performed immunostaining of testicular and epididymal tissue (distal caput, corpus, and cauda) sections with anti-p66 antibodies. A specific staining of the epididymal epithelium was observed along the three regions (Fig. 7a, d, and g), showing a remarkable strong signal on the apical border and stereociliae of principal cells from the distal epididymal caput (see Fig. 7a). The mesenchyme,

FIGURE 6

Effect of sperm proteins p66 and p49 and HSA on sperm–ZP interaction. Spermatozoa were incubated with homologous ZP in the HZA in the presence of 10 μg/mL of sperm proteins p66 (**A**) or p49 (**B**). In all cases, spermatozoa tightly bound to the outer face of each HZ were counted. Results show the number of spermatozoa bound to each HZ (n = 5) (mean ± SD).



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including smooth cells, connective tissue, and endothelial cells, was negative for anti-p66. Sections of testicular tissue showed no specific staining with anti-p66 antibodies (see Fig. 7j). The signal observed was specific, as no staining was obtained when sections were incubated with preimmune serum (see Fig. 7b, e, and h for epididymis, k for testis).

DISCUSSION

Fertilization involves a set of coordinated molecular interactions between the spermatozoa and the oocyte. Our aim is to develop strategies for the identification of surface sperm proteins involved in sperm–egg interaction, and we are mainly interested in those produced by the epididymis. This study describes the identification of a 66-kd peripheral sperm protein of epididymal origin that would participate in sperm recognition of the human ZP.

Of the several approaches widely used for the identification of ZP–sperm receptors, the strategy of the present study involved the isolation of surface sperm proteins by cell treatment with a high ionic strength buffer. Under controlled conditions, this procedure allows removal of peripheral proteins, as has been previously reported (16, 27, 34). In particular, an immune sera obtained using a similar approach recognized epididymal proteins synthesized in response to androgen stimulation (27). With the protocol used in this study, the high percentage of viable sperm found after cell treatment with the buffer suggested a successful extraction of mainly peripheral proteins. Longer incubation times were not carried out because they were associated with a dramatic decrease in sperm viability (data not shown). The protein extract obtained was used to generate the anti-HSE polyclonal antisera, which served as a tool for antigen localization and for functional studies.

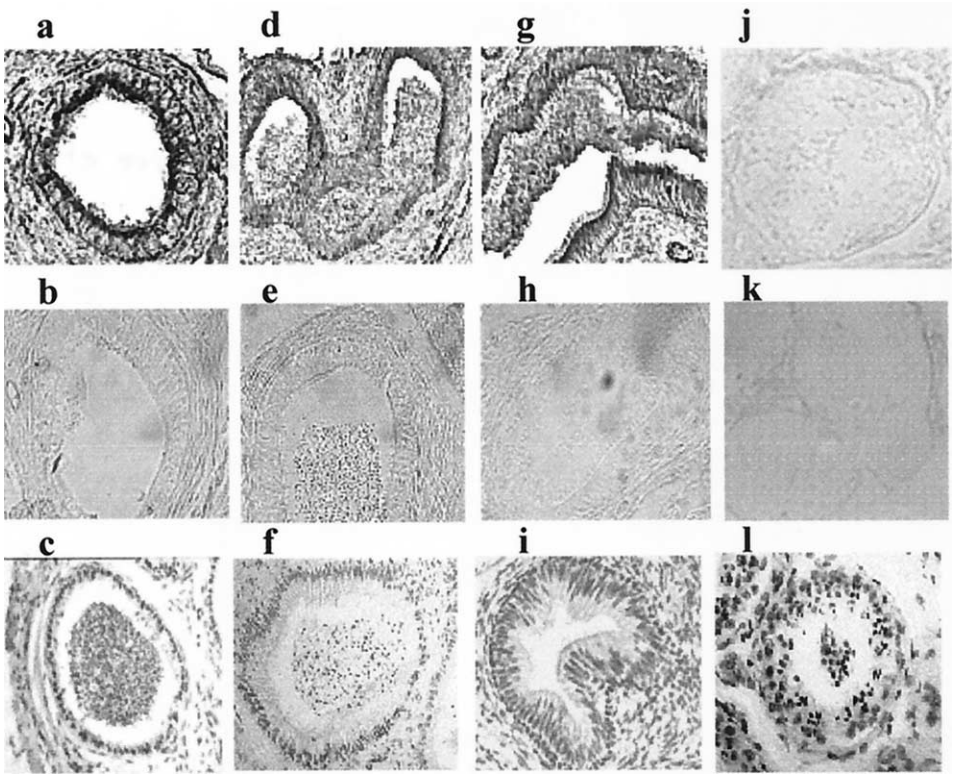
Anti-HSE antibodies recognized antigens in tissue sections from both human testis and epididymis, indicating that sperm proteins produced and secreted by both organs had been extracted. In addition, immunostaining was also found in the sperm acrosomal cap, suggesting that some of the isolated proteins could participate in sperm–ZP interaction. However, due to the complexity of the patterns observed by Western immunoblotting using HSE and protein extracts from tissues and secretions of the male tract, three highly reactive protein regions of 88, 66, and 49 kd abundant components were first selected for analysis. Further characterization was finally done on the last two proteins, because antibodies for p88 were not obtained by adsorption for the immobilized antigen or by animal immunization with the isolated protein (data not shown).

In ejaculated spermatozoa, anti-p66 and anti-p49 reactive proteins were located on the head, over the anterior portion of the acrosomal cap, as observed in other sperm proteins proposed to participate in sperm–ZP interaction (8–11, 13, 15). In cells incubated in vitro under capacitating conditions, some changes in signal localization were observed in both cases. Redistribution of sperm proteins after capacitation has been reported in humans (35–38) and animals (39–49). Some of these polypeptides have been shown to be of epididymal origin, and to participate in sperm–egg interaction (37, 38, 45). Whether changes in localization of p66 and p49 were the result of protein migration or of removal/insertion from tissue secretions remains to be determined.

Localization of p66 and p49 on the sperm head of ejaculated cells, and their permanence in capacitated cells supported a potential role in fertilization. Evaluation of their participation in sperm binding to ZP components was investigated. The decrease in the number of sperm bound seen in the HZA when cells were preincubated using a mixture of antibodies for both proteins suggested a potential role of these proteins in sperm–ZP interaction. Several studies have shown the effectiveness of antibodies for sperm proteins in

FIGURE 7

Immunolocalization of p66 in human epididymis and testis. Tissue sections from distal caput (a, b, c), corpus (d, e, f) and cauda (g, h, i) epididymis, and testis (j, k, l) were immunostained with anti-p66 (a, d, g, j) and preimmune serum (b, e, h, k). Sections stained with hematoxylin (c, f, i, l) are also included (magnification, ×100).



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blocking sperm–oocyte interaction (8, 13, 16, 50). However, a decrease in sperm binding by steric hindrance of the sperm receptor could not be ruled out. Immunoadsorption of anti-HSE against p66 and p49 rendered highly enriched immunoglobulin fractions, but a weak cross reactivity was detected in the anti-p66 fraction with anti-p49 antibodies, and vice versa (data not shown). Thus, the use of the purified proteins instead of each immunoglobulin fraction was considered appropriate in the evaluation of the role of each protein in sperm–ZP binding. Presence of p66 in the HZA had an inhibitory effect upon sperm–ZP interaction, showing a stronger blocking effect than the one observed with the anti-p66 and anti-p49 antibody mixture. In contrast, presence of p49 had no effect on sperm–ZP interaction; thus, no further studies were done with this protein. The decrease in ZP binding was not the result of an inhibitory effect of the antibodies upon sperm motility, because the percentage of motile cells at the end of the incubation period was similar to that before the addition of the immunoglobulins (data not shown).

In addition, the inhibition in the number of sperm bound to the ZP was not caused by a premature sperm acrosome reaction, because gamete incubation was performed in HSM in which calcium ions were replaced by strontium ions. As mentioned, this medium composition has been previously shown to support human sperm capacitation without allowing the induction of the acrosome reaction that may occur in the presence of certain antibodies (33).

The results discussed above suggested participation of p66 in sperm–ZP interaction. Western immunoblot analysis revealed cross reactivity for albumin-like proteins in the p66 region; thus, a possible inhibitory effect of HSA on sperm–ZP interaction had to be discarded. The lack of inhibition observed when the HZA was done in the presence of HSA strongly suggested that a moiety other than HSA was responsible for the inhibitory effect in the incubations done with p66. However, using neither the antibodies nor the protein, the inhibition was total. Similar observations have been obtained when using peptides of ZRK (9) or antibodies for SLIP1-like proteins (16) and P34H (50). It could be speculated that the lack of a complete inhibitory effect would result from inadequate antibody/protein quantities in the gamete assay; alternatively, it could be explained by redun-

dancy of sperm receptors to ensure success in the interaction process (2).

Because sperm–ZP incubation was performed under conditions to prevent the induction of the acrosome reaction, the inhibitory effect observed in sperm preincubated with anti-p66 antibodies would have mainly affected primary sperm interaction between p66 and ZP glycoproteins. Supporting this hypothesis, preliminary studies would suggest the inability of these antibodies to induce the acrosome reaction of fully capacitated sperm (data not shown).

Evidence in favor of the epididymal origin of p66 was provided by the results of the immunohistochemical analysis of tissue sections from the male reproductive tract. The specific staining obtained in sections of the distal caput, corpus, and cauda epididymis would indicate its synthesis along all three segments, though the stronger signal observed in the caput would suggest an increased expression in this region of the organ. Expression of P34H has also been reported along the epididymal duct, restricted to the cell border in the epididymal lumen in association with the microvilli (10, 50). However, P34H mRNA is predominantly expressed in the proximal and distal corpus epididymis (22). The results from the Western immunoblot analysis of protein epididymal extracts done with anti-p66 reinforced the immunohistochemical findings; the signal observed in a 66-kd CEP protein would suggest p66 secretion to the lumen with apparently no further processing. A similar analysis done using anti-p49 antibodies revealed a strong signal with a 49-kd protein in seminal plasma, while the other extracts showed a faint band in the 66-kd region, probably resulting from antibody cross reactivity.

Contrasting with the observations in the epididymis, the consistently negative reaction with testicular sections suggests that p66 epitopes are either absent or not accessible in testicular spermatozoa. In Western blots, a signal observed in testicular proteins with a slightly lower Mr may result from cross reactivity with epitopes exposed after irreversible protein denaturation occurred when placed in sample buffer for protein separation in SDS-PAGE. This signal was not evidenced in the immunohistochemical procedure, because tissue handling may have not involved denaturation of proteins.

In numerous species, the acquisition of sperm fertilizing ability has been associated with changes in the male gamete after spermatogenesis, many of which take place during sperm transit through the epididymis in a process called sperm maturation (for reviews, 51, 52). Secretory proteins produced in the epididymis associated with spermatozoa during transit through the organ would play a key role (for a list of reports, see 53). The existence of a maturational process in the human epididymis has been confirmed (54, 55), and the synthesis and secretion of glycoproteins has been well documented (for a list of reports, see 53). Using a model in which human epididymis responded to androgen stimulation, a group of androgen-dependent proteins were identified (34). Of those, two (Mr 60 and 69) were present in culture media and were detected in CEP, suggesting their secretory nature. Moreover, one (Mr 60) was retained by immobilized immunoglobulins from an antiserum developed for a high salt sperm protein extract (26). Whether p66 is one of those proteins is currently under investigation.

In summary, the present study has described the identification of p66, a human sperm peripheral protein extracted after cell treatment with a high salt buffer, immunolocalized on the sperm head, detected in the proximal segments of the human epididymis and in fluids from the male tract, that would participate in sperm–ZP interaction. Altogether, the results suggest that p66 may be an epididymal coating antigen that would be involved in the acquisition of human sperm fertilizing ability. We are currently performing molecular characterization of p66 by two-dimensional gel electrophoresis and amino acid sequence analysis, as well as by screening of an expression cDNA library from human epididymis recently produced in our laboratory. Assessment of levels of p66 in sperm from subfertile patients known to have a deficient gamete interaction may provide new insights in the diagnosis of male infertility, and eventually may aid in the development of alternative methods for fertility regulation.

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