

# Macaque Sperm Release ESP13.2 and PSP94 During Capacitation: The Absence of ESP13.2 Is Linked to Sperm-Zona Recognition and Binding

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**ABSTRACT** ESP13.2 coats the entire surface of macaque sperm and remains until sperm become capacitated (Yudin et al., 2003: *Biol Reprod* 69: 1118–1128). Capacitation of macaque sperm is synchronized by treatment with dibutyl cAMP (dbcAMP) and caffeine. ESP13.2 and PSP94 constituted ~95% of the proteins released from the sperm surface following treatment with caffeine + dbcAMP. Caffeine and dbcAMP alone induce different patterns of ESP13.2 release. As determined by ELISAs of supernatants and immuno-fluorescent labeling of sperm heads, caffeine alone and caffeine + dbcAMP induced comparable release of ESP13.2, while dbcAMP-treated sperm did not differ from controls. Sperm treated with caffeine + dbcAMP showed a reduction of ESP13.2 from the entire surface, while caffeine treatment alone induced removal of ESP13.2 from the sperm head and midpiece. As confirmed with immunofluorescence, ESP13.2 could be added back to the surfaces of sperm that had been previously exposed to caffeine. Treatment with caffeine significantly increased the number of sperm that bound tightly to the zona pellucida as compared with controls ( $42 \pm 9$  and  $13 \pm 3$  sperm/zona, respectively;  $P \leq 0.01$ ). This increase in binding was inhibited by “adding back” ESP13.2 to the sperm surface ( $12.8 \pm 3$ ;  $P \leq 0.01$ ). Alexa-conjugated anti-ESP13.2 Ig labeling of live sperm showed that only sperm lacking ESP13.2 over the head were capable of tight binding to the zona. Our results suggest that ESP13.2 masks zona pellucida ligands on the sperm surface and its release, as part of capacitation, is required for sperm–zona interaction. *Mol. Reprod. Dev.* 69: 325–337, 2004. © 2004 Wiley-Liss, Inc.

**Key Words:** ESP13.2; macaque sperm; dbcAMP; caffeine; zona pellucida

## INTRODUCTION

Mammalian sperm are structurally complete when they exit the testes, but require epididymal maturation to become fertilization competent (Yanagimachi, 1994; for review). This process, referred to as epididymal

sperm maturation, involves a number of critical alterations to the sperm surface, including enzymatic cleavage of surface proteins, modifications of the glycocalyx, alteration of plasma membrane lipid composition, and the removal and uptake of membrane and membrane absorbed (glyco)proteins (Cooper, 1998; Moore, 1998; Jones, 1999; for review). In vitro fertilization can be accomplished with epididymal sperm, but for in vivo fertilization sperm are transported through the vas deferens and are mixed with the accessory gland secretions at ejaculation. From the time a sperm leaves the testes the sperm surface undergoes a myriad of modifications that are physiologically relevant and necessary to insure successful fertilization (Diekman, 2003). Nevertheless, the functions of many of the sperm-associated proteins produced by the epididymis remain a mystery (Cooper, 1998; for review).

It has been well demonstrated across mammalian species, including primates, that (glyco)proteins produced by the epididymis and/or the accessory glands of the male reproductive tract coat the sperm surface forming a thick glycocalyx that is related to membrane maturation and subsequent fertilization (Schroter et al., 1999). A specific role in capacitation for these surface components, however, has been best described in non-primate species. Shur and Hall (1982) described mouse epididymal factors bearing terminal poly(lactosaminyl) residues that were spontaneously released under capacitating conditions. When added back, these glycoside factors inhibited sperm binding to the zona pellucida by blocking sperm surface galactosyltransferase acceptors. Also in the mouse, Fraser (1984) described another surface component that was lost during capacitation of epididymal sperm, and when added back allowed sperm–zona binding but prevented penetration of the zona

Grant sponsor: Bodega Marine Laboratory, UCal-Davis; Grant number: 2209.

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Received 29 March 2004; Accepted 7 May 2004

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mrd.20132

pellucida and appeared to inhibit the acrosome reaction. This particular factor was later isolated and characterized as a 40 kDa anionic peptide (Fraser et al., 1990). When added to sperm, the peptide fraction stimulated  $\text{Ca}^{++}$  extrusion from the cell, presumably by increasing the activity of plasma membrane  $\text{Ca}^{++}$ -ATPase (Adeoya-Osiguwa and Fraser, 1996). Likewise in the rat, an epididymal secretory protein Crisp-1 is lost from the surface of capacitated sperm. Addition of exogenous Crisp-1 to incubation medium inhibited tyrosine phosphorylation of sperm proteins in a dose dependent manner and thus inhibited capacitation (Roberts et al., 2003). In hoofed animals, a family of secretory proteins, spermadhesins, which are found in the seminal plasma are associated with the sperm surface (Topfer-Petersen et al., 1998). Some of the spermadhesins that are associated with the plasma membrane overlying the acrosome (AQN-1, AQN-2, PSP-1, PSP-2) are lost during capacitation. Spermadhesins appeared to stabilize the acrosome (Topfer-Petersen et al., 1998), and the addition of AQN-1 effectively blocked sperm-oocyte binding (Manaskova et al., 2000).

ESP13.2 is an epididymal secretory protein that is expressed in large quantities in the corpus and cauda epididymidis of the macaques. A highly conserved homologue of this protein has also been identified in human epididymis (Perry et al., 1999; Rodriguez-Jimenez et al., 2003). Recently, it was shown that ESP13.2 coats the entire surface of sperm from the cynomolgus macaque and remains tightly adhered to sperm even after rigorous washing through gradient solutions (Yudin et al., 2003). ESP13.2 is a member of the  $\beta$ -defensin family, a component of the innate immune system (Schutte et al., 2002). Defensins are considered the first line of defense against invading pathogens (Ganz, 2002). Anti-microbial actions have been observed for other  $\beta$ -defensins produced by the epididymis (Hall et al., 2002; Yenugu et al., 2003), but such a function has, as yet, not been demonstrated for ESP13.2. On the other hand, evidence has been presented suggesting that ESP13.2 may be involved or have a role in sperm capacitation (Yudin et al., 2003).

This study describes, for the first time in a primate species, a well characterized surface protein on sperm that disassociates when sperm are capacitated, and when added back, blocks sperm-zona pellucida binding. Specifically, we demonstrate that ESP13.2, as well as tail-associated PSP94, is efficiently removed from the surface of macaque sperm with compounds that synchronize capacitation. Caffeine treatment, in particular, removes ESP13.2 from over the sperm head. Caffeine-induced removal of ESP13.2 leads to enhanced binding of sperm to the zona pellucida, and these effects are completely reversed when free ESP13.2 is added back to the surface of sperm. Furthermore, sperm that bind tightly to the zona pellucida lack ESP13.2 over the anterior head. We conclude that the removal of ESP13.2 from the sperm surface is an essential part of the capacitation process of macaque sperm,

enabling specific binding of macaque sperm to the zona pellucida.

## MATERIALS AND METHODS

### Reagents

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) unless stated otherwise.

### Semen Collection and Sperm Preparation

Animals were housed at the California National Primate Research Center in compliance with the Federal Health Guidelines for Care and Use of Laboratory Animals. Semen samples were collected by electro ejaculation from eight individually caged cynomolgus macaques (Sarason et al., 1991). Each ejaculate was collected into a 15 ml centrifuge tube containing 5 ml of HEPES-buffered Biggers, Whitten and Whittingham (BWW) medium (Irvine Scientific, Santa Ana, CA). After a 1 hr dispersion of the ejaculate in BWW, the samples were checked for motility and only those having greater than 70% motile sperm were used in the experiments. Following removal of coagulum, sperm were pelleted by centrifugation at  $300 \times g$  for further processing. Sperm were then capacitated overnight as described previously (Tollner et al., 2003). Briefly, sperm were washed through a 3.5 ml column of 80% Percoll and suspended in BWW medium containing 30 mg/ml BSA buffered with 35.7 mM sodium bicarbonate ("capacitation medium"). Sperm were washed two more times at  $300 \times g$  and resuspended in capacitation medium to a concentration of  $10\text{--}15 \times 10^6/\text{ml}$ . Following overnight incubation at  $28^\circ\text{C}$  and 5%  $\text{CO}_2$ , sperm were placed into a  $37^\circ\text{C}$  incubator and 5%  $\text{CO}_2$  for an additional 2 hr. This process promotes capacitation in at least a small subset of macaque sperm as determined by the ability of sperm to tightly bind to the zona pellucida and undergo the zona pellucida-induced acrosome reaction. Capacitation of the majority of sperm was synchronized with the addition of 1 mM dibutyryl cAMP (dbcAMP) and 1 mM caffeine ("activators") to sperm suspensions for an additional 1 hr incubation at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  (Tollner et al., 2003). For zona binding studies, sperm were treated with 2  $\mu\text{g}/\text{ml}$  H33348, a fully permeable chromatin dye, during this final capacitation step.

### Preparation of Sperm Surface Released Components

Eleven sperm samples each were washed through Percoll as described above and resuspended in 10 ml of Dulbecco's phosphate buffered saline (DPBS; Life Technologies, Rockville, MD) which lacked energy substrates and BSA. Sperm were washed by centrifugation ( $300 \times g$ ) and the pellet was resuspended to 1–1.5 ml in DPBS. Sperm suspensions ( $50\text{--}60 \times 10^6$ ) were incubated at  $37^\circ\text{C}$  for 1 hr and then treated with activators for an additional hour (see above). Sperm were pelleted at  $\sim 1000 \times g$  for 5 min, the supernatants were then passed through a 0.22  $\mu$  filter and then

concentrated (4–5 $\times$ ) on a Centricon YM-3 (Millipore, Bedford, MA) for 2 hr at 5°C. To insure the removal of activators from the concentrated samples, they were dialyzed into DPBS over 24 hr (4°C) using a Slidealyzer chamber (MWCO 10 kD; Pierce, Rockford, IL). The concentration of proteins released from sperm was determined with a BCA protein analysis system (Pierce). The purity of these sperm proteins was evaluated using an 8–16% tris-glycine gel for electrophoresis (Invitrogen, Carlsbad, CA). The proteins were solubilized in SDS-reducing buffer (Pierce), electrophoresed, and stained as described previously (Yudin et al., 2003). ESP13.2 was identified by probing Western blots with anti-ESP13.2 Ig. The purity of ESP13.2 in the preparation was estimated from the gel according to the optical density of protein bands in the entire gel as determined by analysis of digital scans using NIH ImageJ software and the gel analysis macro. Blank lanes (no protein samples) were set as background and the lanes containing proteins from activated sperm supernatants were compared to the blank lane. Peak areas were measured using the wand tool and each peak was expressed as a percentage of total area under all five peaks. The other prominent protein in the gel was represented as a band that focused at 14 kDa. The sperm proteins in this preparation were the same as those present in the supernatant of capacitated macaque sperm (Yudin et al., 2003). This preparation was used in “add-back” assays, as described below.

### Antibody Production and Preparation

Antibodies were developed to the purified ESP13.2 as described previously (Yudin et al., 2003). Sperm were washed through 80% Percoll, resuspended in DPBS, pelleted by centrifugation (300  $\times$  g) for 10 min, and treated with PI-PLC 3 U/80  $\times$  10<sup>6</sup> sperm. Sperm samples were incubated at 37°C. The supernatant was passed through a 0.22  $\mu$ m syringe filter, concentrated, and electrophoretically separated on an 8–16% gel. The gels were stained with Gel Code Blue (Pierce). For ESP13.2 collection, the 53 kDa band was cut from the gel and electro-eluted (Yudin et al., 2003). From the same gels, the 14 kDa protein band was collected similarly. After complete electro-elution, samples were chemically reduced with 0.1 M dithiothreitol (DTT) and electrophoresed on a 16% gel. The entire 31–35 kDa band was cut from the gel and electro-eluted for immunization. The 14 kDa band was prepared in like fashion. After electro-elution, the 14 kDa protein was concentrated and 25  $\mu$ g aliquots, mixed with Freund's complete adjuvant for the first injection. The three subsequent injections were done with Freund's incomplete adjuvant for a total of four immunizations into three separate chickens. After the fourth injection, a series of eight eggs were collected and the IgY was purified using the reagents and procedures outlined in Eggcellent (Pierce). Development of antibodies to ESP13.2 in rabbits was thoroughly described previously (Yudin et al., 2003).

Antibodies were also developed to the sperm surface by fixing sperm in 1.5% para-formaldehyde/0.15%

glutaraldehyde in DPBS before and after treatment with activators. Sperm (20  $\times$  10<sup>6</sup>) were fixed for 1 hr and then thoroughly washed in DPBS. A single injection was given to rabbits with complete Freund's adjuvant and Ig levels were monitored weekly.

All serum samples were initially heat inactivated (56°C/30 min) and then precipitated with ammonium sulfate (0.24 g/ml). The ammonium sulfate was added slowly over a 4 hr period at 4°C. The precipitated Ig was pelleted and resuspended in DPBS and dialyzed overnight. The Ig was stored at –20°C.

### Characterization of 14 kDa Protein

The 14 kDa protein was purified using two different procedures. The product of each method was submitted for sequencing to separate labs. The 14 kDa protein purified after PI-PLC treatment was cut out of gels (16%) and electro-eluted. The eluted protein was reduced with 100 mM DTT and reloaded on a 16% gel. The gel was stained with Gel Code Blue (Pierce). The 14 kDa protein was submitted to the W.M. Keck Biotechnology Resource Laboratory (Yale University). The activator released surface components were concentrated 4–5 $\times$ , solubilized in buffer (Pierce), and run on a 8–16% gel. The 14 kDa band was cut out and submitted to the Molecular Structure Laboratory, University of California, Davis. Sequencing was done on an ABI Procise Protein Sequencing System. All chemistry programs and data collection/analysis were performed using standard Procise protocols. The individual amino acids were cleaved from the protein using standard Edman chemistry and resolved on the standard ABI RP columns using standard solvents. Absorbance was monitored at 269 nm; results were displayed and reports were generated using the ABI 610a V.2.1 software running on a Mac G3. The sequence data were compared to the existing DNA data banks for any sequence similarity.

Tissue samples were acquired from adult cynomolgus macaques at the time of necropsy. Small portions of the brain, kidney, heart, muscle, testes, ovary, bladder, liver, spleen, and prostate were solubilized in 0.3 M TRIS, 5% SDS, and 50% glycerol. Male reproductive tissue caput, corpus, caudal, vas deferens, seminal vesicles, and prostate were solubilized as previously mentioned. To obtain seminal fluid the ejaculate was suspended in 1 ml of Hepes-buffered BWW and allowed to sit for about 10–15 min. The 1 ml sperm suspension was centrifuged at 1000  $\times$  g and the supernatant was filtered (0.2  $\mu$ m), prior to the addition of SDS-solubilizing buffer (Pierce). DTT (10 mM) was added and the sample boiled for 3 min.

Ejaculated sperm suspensions were pelleted (300  $\times$  g) for 10 min, resuspended in 1 ml of DPBS and layered over a 3.5 ml column of 80% Percoll in DPBS. After centrifugation for 30 min at 300  $\times$  g, the resulting pellet was washed twice in DPBS (10 ml). Washed sperm were resuspended in 1 ml of boiling nonreducing SDS-solubilizing buffer (Pierce). After 2 min of boiling, the sample was centrifuged (1000  $\times$  g) and the resulting

supernatant was chemically reduced (100 mM DTT) and boiled an additional 2 min.

Gel electrophoresis and Western blots were prepared as described previously (Yudin et al., 2003). Briefly, after electrophoresis, the gel was electroblotted to nitrocellulose membranes and blocked for at least 2 hr in TBS (50 mM Tris-HCl, pH 7.4; 0.3 M NaCl) containing 5% nonfat dry milk and 0.1%  $\text{NaN}_3$ . After blocking, the blots were incubated with 50  $\mu\text{g}$  of Ig per 10 ml of TBS with 3% BSA and 0.1%  $\text{NaN}_3$ . After thorough washing in TBS (50 mM Tris-HCl, pH 7.4; 0.3 M NaCl), blots were subsequently incubated with the appropriate secondary antibody (1:2000), goat anti-rabbit IgG-alkaline phosphatase (BioRad; Richmond, CA) or goat anti-chicken IgG-alkaline phosphatase (Aves Labs, Tigard, OR). After washing in TBS, immune complexes were detected using precipitating alkaline phosphatase substrate (1-Step NBT-BCIP, Pierce).

#### Fluorescent Immunolocalization of ESP13.2 and the 14 kDa Protein

Sperm were fixed in 2% paraformaldehyde in DPBS for 20 min. After fixation, sperm were thoroughly washed (2–3 $\times$ ) in blocking solution (1% BSA, 0.1%  $\text{NaN}_3$ , 1% gelatin/DPBS). Sperm samples were suspended in either anti-ESP13.2 Ig or anti-14 kDa Ig prepared as above (10  $\mu\text{g}$  Ig/ml), gently rolled for 1 hr, and then washed 3 $\times$  in blocking solution and resuspended in a solution of 20  $\mu\text{g}/\text{ml}$  goat anti-rabbit or goat anti-chicken Alexa 488 (Molecular Probes, Eugene, OR) in blocking solution. The samples were again rolled for 1 hr and then thoroughly washed and resuspended in a fluorescent stabilization medium (50% glycerol, 0.2%  $\text{NaN}_3$ , 1% paraformaldehyde/DPBS). Photomicrographs were taken of representative cells using a cooled CCD digital camera (Magnafire; Optronics, Santa Barbara, CA) mounted on a Leitz Laborlux S microscope (Carl Zeiss Vision, GmbH, Germany) equipped with 200W mercury fluorescence vertical illuminator and a 1-Lambda Ploemopac incident light fluorescence illuminator employing an I3 filter cube with a BP 450–490 excitation filter, a RKP 0510 dichromatic mirror, and a LP 515 suppression filter. Optics included a 3.3X intraocular magnifier (Scientific Instruments, Sunnyvale, CA) and a Zeiss 63X oil emersion fluorescence objective (JH Technologies, San Jose, CA). Initial images were captured using Magnafire 2.0 software (Optronics) and processed with Adobe Photoshop (Adobe Systems, San Jose, CA) for production of figures.

#### Pixel Analysis of Fluorescently Labeled Sperm

Ten to 15 microscopic fields of labeled sperm per treatment were randomly captured for pixel analysis with the microscope imaging system described above. Each field contained on average from three to five sperm that did not have debris or other sperm overlapping the head. The images of these sperm heads were digitally cropped with a standard ovoid stencil using Adobe Photoshop and placed in a composite file for pixel analyses. Images containing 36 sperm heads were

thresholded to the same level for all treatments and average pixel area and gray values determined using MetaMorph 6.1 Image Analysis (Universal Imaging Corp., Downingtown, PA) software. The values for 36 sperm from each treatment were pooled across each experiment. A different male was used in each experiment.

#### Effects of Caffeine and dbcAMP on Loss of ESP13.2 From the Sperm Surface

Following Percoll washing, overnight incubation at RT, and 2 hr at 37.5°C as described above, sperm were treated with 2 mM caffeine, 2 mM dbcAMP, or 1 mM caffeine + 1 mM dbcAMP for 1 hr. Control sperm preparations received neither compound. Sperm were washed free of supernatants, resuspended in warm DPBS, and checked for survival. No differences in sperm motility due to treatment were observed. Motility across all treatments was  $>70 \pm 7\%$  (mean  $\pm$  SEM). Sperm were labeled immuno-fluorescently, and assessed for localization of ESP13.2 and the 14 kDa protein as described above. The experiment was repeated 5 times, each time with sperm from a different male.

Following treatment supernatants were filtered and then pre-incubated with rabbit anti-ESP13.2 Ig (25  $\mu\text{g}/\text{ml}$ ) for 1 hr at 37°C. The amount of free anti-ESP13.2 Ig in each supernatant was determined by ELISA on micro titer plates with wells coated with 5  $\mu\text{g}/\text{ml}$  of purified ESP13.2. Coated wells were blocked, washed, and incubated with supernatants containing anti-ESP13.2 Ig for 1 hr. Wells were washed again, and incubated with biotinylated anti-rabbit Ig (secondary antibody) suspended in blocking solution for 30 min. After washing, an avidin and biotinylated horseradish peroxidase complex (ABC kit) was added and allowed to incubate for 30 min. Following removal of unbound peroxidase by thorough washing, the chromatic substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was added to the wells for 30 min. The optical density (o.d.) of the green colored reaction product was measured with a micro titer plate reader at 405 nm. Percent inhibition of anti-ESP13.2 Ig absorption to the wells was calculated as 1-o.d. of treatment/o.d. of control (anti-ESP13.2 Ig in capacitation medium only). The experiment was repeated 6 times, each time with sperm from a different male.

#### Detection of ESP13.2 After Recombination With Sperm (Add-Back Assay)

Sperm samples from three males were washed through Percoll, incubated overnight at RT, transferred to 37°C, and treated with 2 mM caffeine or with an equivalent volume of DPBS as described above. Sperm were then washed by centrifugation and sperm pellets were resuspended to give a final concentration of  $10 \times 10^6/\text{ml}$ . Caffeine-treated aliquots of sperm were further divided. To one aliquot, one part of ESP13.2 solution was combined ("add back") with four parts sperm suspension ( $\sim 7 \mu\text{g}/\text{ml}$  ESP13.2). To the other aliquot, the same volume of DPBS containing 35  $\mu\text{g}/\text{ml}$  BSA was added (control). After 1 hr at 37°C and 5%  $\text{CO}_2$ ,

sperm were washed again by centrifugation and resuspended in capacitation medium to  $\sim 10 \times 10^6$ /ml. The control, caffeine, and "add back" treatments were processed for immuno-fluorescent labeling and pixel analysis with anti-ESP13.2 and anti-14 kDa Ig.

### Effect of Recombined ESP13.2 on Sperm Binding to the Zona Pellucida

Sperm were washed and incubated overnight to induce capacitation as described above. Following 2 hr incubation at 37°C and 5% CO<sub>2</sub>, sperm suspensions were divided into three aliquots. Two of the aliquots were treated with 2 mM caffeine and the remaining aliquot with an equal volume of DPBS. After 1 hr, treated sperm were washed 1× at 300 × g and resuspended in capacitation medium. One of the caffeine-treated aliquots received the "add back" solution ( $\sim 7$  μg/ml ESP13.2), the other two aliquots (caffeine-treated and control) received an equal volume of DPBS containing BSA. Treated sperm were incubated an additional hour, washed 1× at 300 × g, and resuspended in capacitation medium for assessment of zona pellucida binding and motility.

Acrosome reactions were detected in sperm suspensions prepared for zona binding according to the methods described by (Cross et al., 1988). Briefly, sperm were dried onto glass slides and stained with 100 μg/ml FITC-PSA in DPBS. Excess FITC-PSA was removed by rinsing slides with dH<sub>2</sub>O. Anti-fading solution (DPBS containing 32% glycerol, 0.5% paraformaldehyde, and 0.01% sodium azide) was deposited over regions of dried sperm and overlaid with a glass cover slip. Sperm were observed with a Lietz Laborlux S microscope equipped as described above. 200–300 sperm from each treatment were scored for presence of the acrosome.

### Sperm-Zona Pellucida Binding Assay

Ovaries were obtained at necropsy from adult female cynomolgus macaques at the CRPRC. Zona pellucida-intact immature oocytes were collected from the ovaries and were frozen at –80°C in 2 M dimethyl sulfoxide (DMSO) in DPBS according to previously published protocols (VandeVoort et al., 1992). The oocytes were thawed at 22°C and rinsed through three dishes, each containing 0.5 ml of Hepes-buffered BWW medium, to remove DMSO prior to experiments. Two zona were transferred with a fine bore glass pipette into sperm suspensions maintained under equilibrated mineral oil (37°C and 5% CO<sub>2</sub>) and allowed to co-incubate for 2 min. After co-incubation, zona were transferred through three rinse drops of Hepes-buffered BWW (two aspirations per drop) to remove loosely or non-specifically bound sperm (VandeVoort et al., 1992). Zona were then placed on a glass slide along with 5 μl of medium between four posts of silicon grease containing 100 μ glass beads. A glass coverslip was added and the number of zona-bound sperm was scored by visualizing the Hoechst stain with the 50× fluorescence objective of a Lietz Laborlux S microscope (Carl Zeiss Vision) equipped with 200 W mercury fluorescence vertical illuminator and a

1-Lambda Ploemopac incident light fluorescence illuminator employing an A filter cube with a BP 340–380 excitation filter, a RKP 0400 dichromatic mirror, and a LP 425 suppression filter.

### Assessment of ESP13.2 on Sperm Bound to the Zona Pellucida

Sperm were capacitated as described above but not treated with activator(s). Prior to zona pellucida binding, sperm were pre-incubated live with Alexa 488 conjugated-anti-ESP13.2 Ig at 20 μg/ml. Coupling of Alexa 488 reactive dye to anti-ESP13.2 Ig was accomplished using the Alexa 488 Protein labeling kit (Molecular Probes). After 1 hr sperm were washed 1× at 300 × g and resuspended in capacitation medium free of Ig. Sperm were incubated an additional hour and washed. Motility was assessed and zona pellucida binding was performed as described above. Antibody labeling of sperm in suspension droplets and sperm attached to the zona was assessed as described above in both live and fixed sperm preparations.

### Statistical Analysis

Statistical analyses were conducted using one-way analysis of variance followed by Duncan range testing. Values are given as mean ± SEM. *AP*-value  $0 \leq 0.01$  was considered significant.

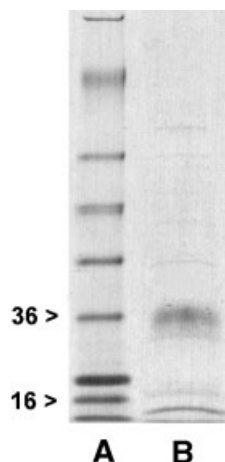
## RESULTS

### Characteristics of the ESP13.2 Preparation

It was previously demonstrated that when macaque sperm were treated with activators there was a significant loss of ESP13.2 (Yudin et al., 2003). However, following overnight incubation to induce capacitation, treatment with activators often resulted in a decrease in sperm survival, which clouded the issue of ESP13.2 release, since release of surface proteins could occur post mortem. In preliminary experiments, we determined that the sperm proteins released into the incubation medium could be more reliably measured if sperm were incubated for a shorter period of time in DPBS before treatment with activators. After incubation under these conditions, a large band at 32–36 kDa and a thinner band at 14 kDa were consistently the prominent proteins in the supernatant (Fig. 1). Analysis of digital images of gels following electrophoresis of these supernatants indicated that these two proteins comprise approximately 95% of the total released protein, with the 32–36 kDa band representing 74.7% of the total amount and the 14 kDa band representing 19.9%.

### Amino Acid Sequences of the 32–36 kDa and 14 kDa Sperm Proteins

At least two peptide fragments were generated from the tryptic digest of the 14 kDa protein isolated from gels. After Edman degradation and sequence analysis, it was determined that the activator-released 14 kDa peptide was 100% homologous with prostate secretory protein PSP94 (Nolet et al., 1991; Fig. 2). This same



**Fig. 1.** Sperm samples were washed by centrifugation through Percoll and phosphate buffered saline and resuspended to 1–1.5 ml in DPBS. Sperm suspensions were incubated at 37°C for 1 hr and then treated with activators caffeine and dibutyl cAMP (dbcAMP) for an additional hour. Sperm were pelleted by centrifugation, and the supernatants were filtered, concentrated, and dialyzed. Sperm proteins in the supernatants were solubilized in SDS-reducing buffer and were separated on 8–16% tris-glycine gels for electrophoresis. Two dominant proteins were seen to be released from sperm with activators (**Lane B**); one at 32–36 kDa and one at 14 kDa as determined from the molecular weight standard (**Lane A**). Analysis of the relative optical density of the protein bands in the activator released material was indicated with the 32–36 kDa protein and 14 kDa protein represented approx. 75 and 20%, respectively, of the total protein observed in the gel.

protein had previously been shown to be released during PI-PLC treatment. Antibodies were developed to the PSP94 derived from PI-PLC treatment. From the same gel, the 31–36 kDa band was also cut out, trypsin digested, and sequenced. Each of the peptide fragments had 100% homology with ESP13.2, a previously described macaque sperm surface (glyco)protein (Yudin et al., 2003).

### Western Blot Analysis

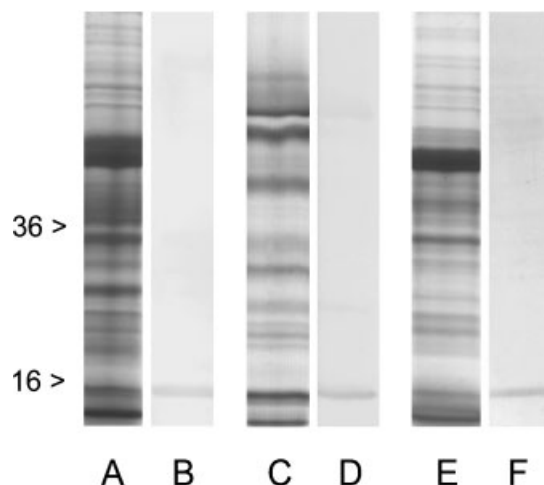
It has been well-documented that PSP94 is produced primarily by the prostate gland of both rodents and primates (Akiyama et al., 1985; Thota et al., 2003) and is found in human seminal plasma (Ohkubo et al., 1995). The specificity of our antibody to PSP94 was verified by probing whole macaque prostate gland preparations in

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1  ★ SCSFIPNERF PGDSTR□ECTD LKGNKHPINS KWKTD★CERC
50  ★ TCYK/TEII/CC TLIATPVGYD KKKCQRIFRK EDCK/YIVVEK/
80  KNP★KTCPID QWIL

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**Fig. 2.** The 12–14 kDa protein band released from sperm with activator was isolated, purified and subjected to enzymatic cleavage, peptide purification, Edman degradation, and amino acid identification. Sequence of two fragments (underlined sections) had 100% amino acid identity with a previously described prostate secretory protein, PSP94. Stars indicate cysteine residues, open squares indicate negatively charged residues (aspartic and glutamic acid), and closed squares represent positively charged residues (arginine, lysine). The entire protein has a net +7 charge.



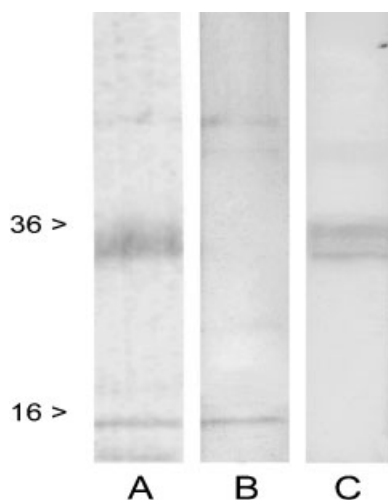
**Fig. 3.** Antibodies developed in rabbits to purified PSP94 were used to probe extracts from the prostate (**A**), seminal plasma (**C**), and whole cynomolgus sperm (**E**). All extracts were SDS-solubilized but not reduced and separated by electrophoresis on 16% tris-glycine gels. When blotted to nitrocellulose and probed with anti-PSP94 Ig a single prominent immunoreactive band was revealed in prostate (**B**), seminal plasma (**D**), and whole sperm (**F**), each with a molecular weight of 14 kDa, consistent with the previous determinations for PSP94.

Western blot analyses (Fig. 3). Antibodies raised against the 14 kDa protein recognized a single protein, represented by a concise band with an approximate molecular weight of 14 kDa. (Fig. 3). The anti-14 kDa Ig did not recognize any component of a Western blot of tissue from the brain, bladder, heart, muscle, kidney, liver, ovary, spleen, or lung. Ig probe of blots of extractions of the male reproductive tract (testes, caput, corpus, and caudal epididymis, vas deferens, seminal vesicles, and prostate glands) resulted in one immunoreactive band at 14 kDa found only in the prostate extraction (Fig. 3B). Similarly, this antibody recognized one protein of the same molecular weight on blots of whole macaque sperm (Fig. 3F) and a single band in the seminal fluid preparation (Fig. 3D).

Western blots of activator-released sperm proteins were probed with anti-ESP13.2 and anti-PSP94 Igs. As with whole sperm blots (Fig. 3F), anti-PSP94 Ig recognized a single protein band at 14 kDa (Fig. 4B). Anti-ESP13.2 Ig immunoreacted with a single band at 32–36 kDa (Fig. 4C), a result consistent with recognition of the same Ig of ESP13.2 on blots of epididymal tissue, whole sperm and PI-PLC released sperm protein (Yudin et al., 2003).

### Fluorescence Localization of PSP94

In agreement with Western blots demonstrating that PSP94 is released from the sperm surface, anti-PSP94 Ig intensely labeled the majority of macaque sperm that were fixed prior to labeling. Prior to being washed through Percoll, labeling of most sperm was intense over the principle piece of the flagellum with faint labeling over the sperm head. After Percoll washing, the labeling was restricted to the principal piece of the flagellum (Fig. 5B). Approximately 15% of sperm either lacked

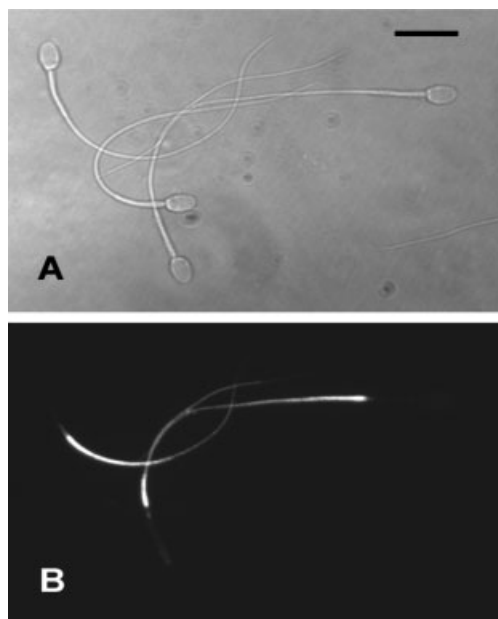


**Fig. 4.** Activator-released proteins were separated by electrophoresis on a 16% tris-glycine gel (A) and blotted onto nitrocellulose. Blots were probed with anti-PSP94 Ig (B) and anti-ESP13.2 Ig (C).

label or had only faint labeling over the flagellum after sperm had been thoroughly washed through an 80% Percoll column and washed (2 $\times$ ) before resuspending in capacitation buffer.

#### ESP13.2 Loss After Treatment With Caffeine and/or dbcAMP

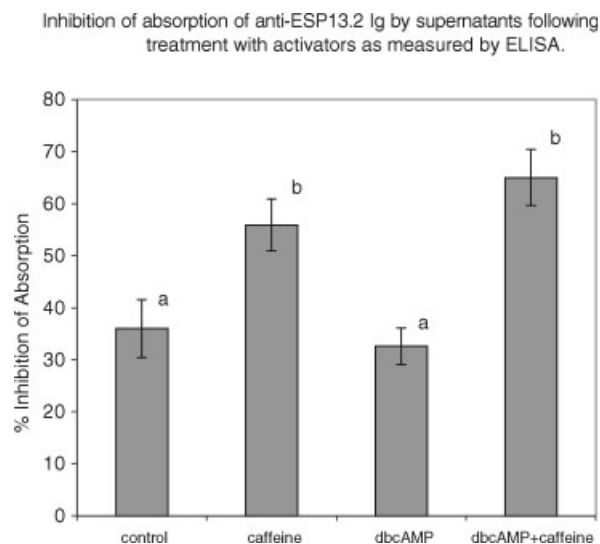
Successful *in vitro* fertilization with macaque gametes has long been known to require treatment of sperm with both dbcAMP and caffeine (Bavister et al.,



**Fig. 5.** Immunofluorescent labeling of PSP94 on macaque sperm. Sperm washed through Percoll and incubated overnight before fixation and antibody labeling with anti-PSP94 Ig. Approx. 85% of sperm labeled brightly over the principle piece of the flagellum (B), the remaining sperm had little to no labeling. Corresponding bright-field image shown in (A). Micrograph is representative of sperm observed from three different males. Bar = 10  $\mu$ m.

1983). Recent studies have shown that such treatment synchronizes capacitation of macaque sperm (Tollner et al., 2003). When used together, treatment with these compounds results in an accelerated increase in sperm binding to the zona pellucida followed by immediate induction of the acrosome reaction (VandeVoort et al., 1992; Tollner et al., 2003). Separately, these compounds have very different effects on sperm. Caffeine treatment enables sperm to readily bind to the zona but these sperm do not undergo the acrosome reaction. On the other hand, only low numbers of dbcAMP-treated sperm can initiate binding to the zona, but a high percentage of those sperm undergo the acrosome reaction (VandeVoort et al., 1994). Given these differences, we investigated whether ESP13.2 loss from the sperm surface would take place following treatment with either compound and/or whether both of the compounds are required.

ESP13.2 was detected by ELISA in supernatants following all treatments including controls (Fig. 6). The relative amount of ESP13.2 released into the supernatant was inferred from the percent inhibition of absorption of anti-ESP13.2 Ig to the micro titer plate. The greatest amount of ESP13.2 was released following treatment with both caffeine and dbcAMP. Caffeine treatment alone also induced a significant release of ESP13.2 that was comparable to treatment with both compounds. When response was normalized to control treatment values, treatment with both compounds



**Fig. 6.** Following overnight incubation in capacitation medium at RT, sperm were incubated for 3 hr at 37°C and treated with activators (DPBS = control; 2 mM caffeine, 2 mM dbcAMP, and 1 mM caffeine + 1 mM dbcAMP) for 1 hr. Sperm were pelleted and supernatants were pre-incubated with rabbit anti-ESP13.2 Ig for 1 hr at 37°C. The amount of free anti-ESP13.2 Ig in each supernatant was determined by ELISA on microtiter plates with wells coated with purified ESP13.2. Bars represent the percent inhibition of anti-ESP13.2 Ig absorption to the wells which is proportional to the amount of ESP13.2 released from sperm into the supernatants. Error bars represent the standard error of means. Different letters indicate significant differences between treatments ( $P \leq 0.01$ ). Experiment was repeated 6 times, each time with a different male.

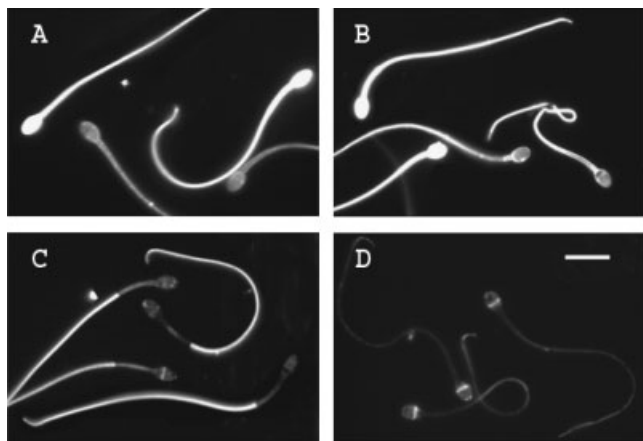
released 31.4% more ESP13.2 into the supernatant than did treatment with caffeine alone ( $P \leq 0.05$ ). Supernatants of both control and dbcAMP-treated suspensions were indistinguishable in the amount of ESP13.2 released, and had significantly less ESP13.2 than either caffeine or caffeine + dbcAMP treatments (Fig. 6;  $P \leq 0.01$ ).

Localization of ESP13.2 on the surface of sperm in these suspensions differed according to the treatment. The majority of control sperm and dbcAMP-treated sperm had bright uniform fluorescence over the entire head and flagellum following labeling with anti-ESP13.2 Ig (Fig. 7A,B). Sperm treated with both caffeine and dbcAMP showed a loss of ESP13.2 over the entire surface, with a faint but distinguishable band of fluorescence remaining over the equatorial segment of most sperm (Fig. 7D). Sperm treated with caffeine alone had a loss of fluorescence primarily over the head and midpiece with little to no loss over the flagellum (Fig. 7C). Loss of labeling over the head did not result from the acrosome reaction of treated sperm because reacted sperm comprised less than 2% of the population in all treatment groups.

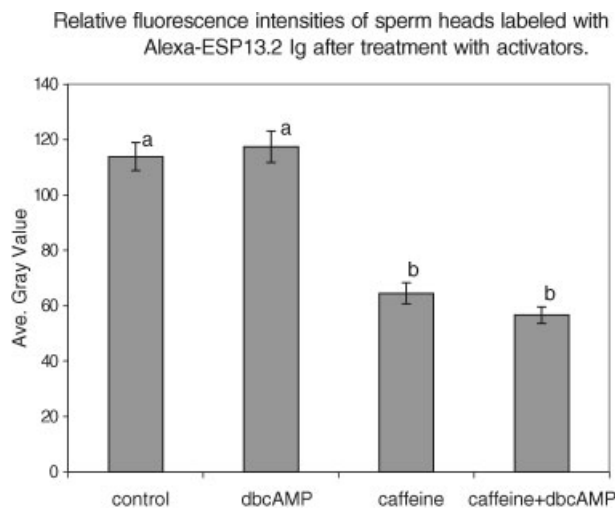
Differences in label intensity over the heads of sperm from each treatment were detected using pixel analyses of images collected under identical microscope and camera parameters. In general, sperm treated with caffeine or with caffeine + dbcAMP had a 50% reduction in fluorescence over the head as compared to control sperm (Fig. 8;  $P \leq 0.001$ ). The fluorescence of control sperm and dbcAMP-treated sperm was indistinguishable.

#### Detection of ESP13.2 on the Sperm Surface Following Add-Back

Time dependent, capacitation-related changes in the location of ESP13.2 have been demonstrated previously by indirect immuno-fluorescence (Yudin et al., 2003).



**Fig. 7.** Immunofluorescent labeling of ESP13.2 after treatment with activators. Following overnight incubation in capacitation medium at RT, sperm were incubated for 3 hr at 37°C and treated with activators. Control sperm (A), sperm treated with 2 mM dbcAMP (B), sperm treated with 2 mM caffeine (C), and sperm treated with 1 mM of both dbcAMP and caffeine (D) were fixed and labeled with anti-ESP13.2 Ig. Bar = 10  $\mu$ m.



**Fig. 8.** Assessment of immunofluorescent labeling intensity of the heads of sperm after treatment with activators (DPBS = control; 2 mM caffeine, 2 mM dbcAMP, and 1 mM caffeine + 1 mM dbcAMP). Immunofluorescent images of sperm heads labeled with anti-ESP13.2 Ig were thresholded to the same level for all treatments and average pixel area and gray values determined for each sperm head using MetaMorph Image Analysis software. The values for 36 sperm from each treatment from each experiment were pooled over four experiments, each experiment performed with sperm from a different male. Bars represent the mean pixel intensity of sperm heads with treatment. Error bars represent the standard error of means. Different letters indicate significant differences between treatments ( $P \leq 0.01$ ).

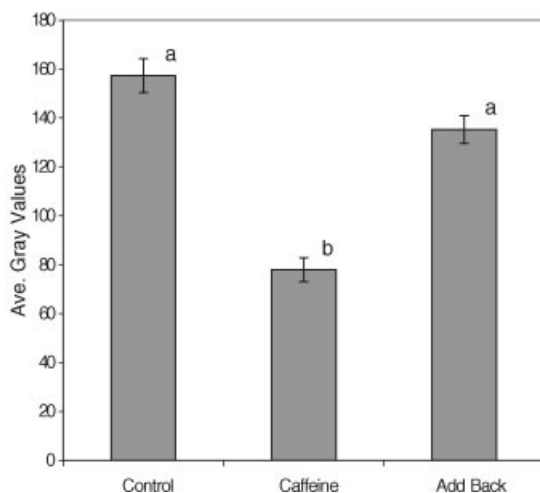
The same approach was used in the present study to determine if ESP13.2 could be added back to the surface of sperm following removal from the sperm head by caffeine treatment. As in the previous experiment, the majority of caffeine-treated sperm had very little or no ESP13.2 over the sperm head, except on the equatorial segment. Following incubation with the ESP13.2 preparation and light washing by centrifugation, a coating of ESP13.2 on the anterior head of caffeine-treated sperm was detected by immuno-fluorescence. Differences in head labeling according to treatment were detected by pixel analysis (Fig. 9). Sperm treated with the ESP13.2 preparation had more fluorescence over the head than caffeine-treated sperm (Fig. 7;  $P \leq 0.05$ ). The brightness of sperm treated with the ESP13.2 preparation was statistically indistinguishable from that of control sperm, which had not been exposed to caffeine.

Indirect immunofluorescence using anti-PSP94 Ig revealed labeling only along the principal piece of the flagellum. No label was observed on the sperm head (not shown). No difference in labeling intensity was observed after sperm were treated with caffeine or after caffeine-treated sperm were treated with the ESP13.2 preparation (data not shown).

#### Sperm Binding to the Zona Pellucida Following Treatment With ESP13.2 Preparation

Caffeine-treated sperm were used to assess the effect of treatment with the ESP13.2 preparation on sperm-zona binding. Caffeine treatment of sperm results in high numbers of sperm binding to the zona pellucida,

Relative fluorescence intensities of sperm heads labeled with Alexa-ESP13.2 Ig after treatment with caffeine and "add back" solution.



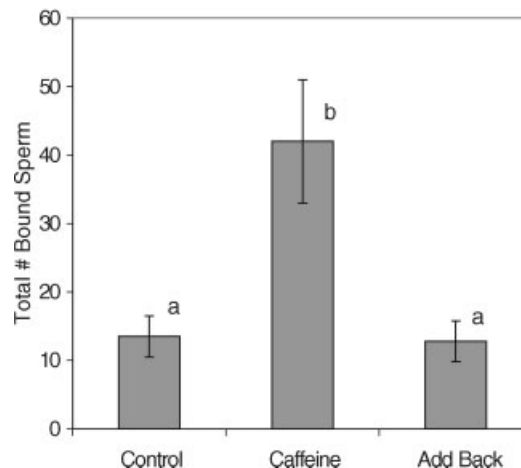
**Fig. 9.** Assessment of immunofluorescent labeling intensity of the heads of sperm after treatment with 2 mM caffeine and caffeine-treated sperm following addition of add back solution containing ESP13.2. Immunofluorescent images of sperm heads labeled with anti-ESP13.2 Ig were thresholded to the same level for all treatments and average pixel area and gray values determined for each sperm head using MetaMorph Image Analysis software. The values for 36 sperm from each treatment from each experiment were pooled over three experiments, each experiment performed with sperm from a different male. Bars represent the mean pixel intensity of sperm heads with treatment. Error bars represent the standard error of means. Different letters indicate significant differences between treatments ( $P \leq 0.05$ ).

but caffeine-treated sperm do not acrosome react after zona binding (VandeVoort et al., 1994). In the present study, fewer than 2% of caffeine-treated sperm in suspension were acrosome reacted and zona-bound sperm were never observed undergoing the acrosome reaction (data not shown). These conditions enabled observation of primary sperm-zona binding and avoided the loss of zona bound sperm that can follow the acrosome reaction (Tollner et al., 2003). As observed previously, caffeine induced a three to four fold increase in the number of sperm tightly bound to the zona pellucida compared to controls (Fig. 10;  $P \leq 0.01$ ). Caffeine-treated sperm that were treated with the ESP13.2 preparation bound to the zona in significantly lower numbers that were indistinguishable from controls (Fig. 10).

#### Assessment of ESP13.2 on Sperm Bound to the Zona Pellucida

Live sperm labeled with Alexa 488-conjugated anti-ESP13.2 Ig were observed during sperm-zona binding. Capacitated sperm were used without activator treatment because the majority (75%) of sperm in these suspensions had moderate to bright labeling of ESP13.2 over the head (Fig. 11A,B). Very few sperm ( $6 \pm 1\%$ ) that were bound to the zona had labeling over the anterior head and these were always faintly labeled (Fig. 11E,F; sperm 2). The majority of bound sperm ( $94 \pm 5\%$ ) either lacked labeling over the anterior head with some equatorial segment labeling (Fig. 11C,D sperm 2 and 3

Effect of adding back ESP13.2 to caffeine treated sperm on sperm-zona binding. N=7.

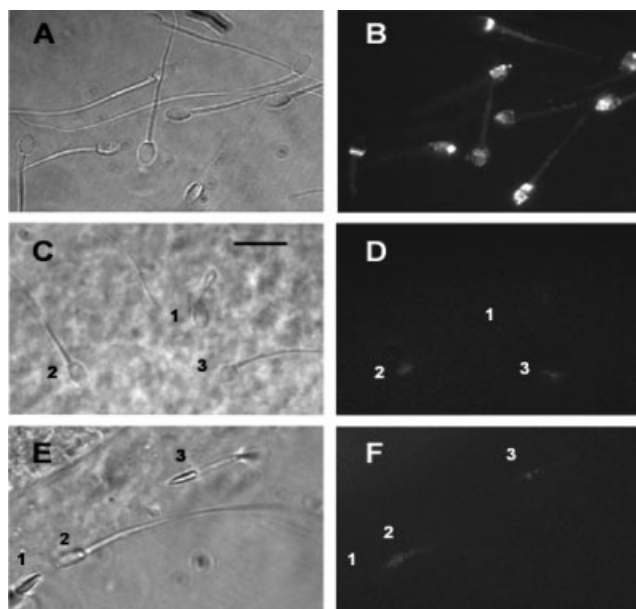


**Fig. 10.** Zona pellucida binding assay with sperm treated with 2 mM caffeine and caffeine-treated sperm following addition of add back solution containing ESP13.2. Bars represent averages of the total number of sperm bound per zona with treatment. Experiment was repeated 7 times, each time with sperm from a different male. Error bars represent the standard error of means. Different letters indicate significant differences between treatments ( $P \leq 0.01$ ).

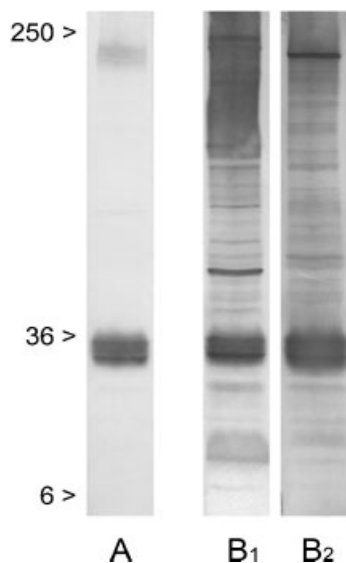
and Fig. 11E,F sperm 3) or lacked label altogether over the head (Fig. 11C,D, sperm 1 and Fig. 11E,F, sperm 1).

#### Assessment of Role of Surface Coat in Immunologic Protection

Prior to injection into rabbits, sperm were fixed either before activation, preserving the surface coat along the



**Fig. 11.** Live sperm were labeled with Alexa 488-conjugated ESP13.2 Ig prior to sperm-zona binding. Immunofluorescence of sperm in suspension around the zona is shown in (B). Immunofluorescence of sperm bound to the zona are shown in (D) and (F). (A), (C), and (E) are the corresponding bright-field images. Bar = 10  $\mu$ m.



**Fig. 12.** Proteins from whole sperm extracts were separated by gel electrophoresis and blotted onto nitrocellulose. Blots were probed with serum from rabbits injected with sperm that were not activated (ESP13.2 was intact; **A**) and sperm that were activated (ESP13.2 was removed; **B<sub>1</sub>** and **B<sub>2</sub>**).

sperm membrane, or after activation, in which the majority of ESP13.2 is lost. Sera from rabbits 1 month after the injections recognized various protein bands on Western blots from whole sperm extracts (Fig. 12). Serum from a rabbit injected with non-activated sperm recognized only two protein bands, one at ~220 kDa and one at 32–36 kDa (Fig. 12A). The latter band was more intensely labeled and corresponds in mobility to ESP13.2. Sera from two rabbits that received activated sperm injections recognized a host of proteins (>100) on whole sperm blots (Fig. 12B<sub>1</sub>,B<sub>2</sub>).

### DISCUSSION

Sperm for the most part exit the testes essentially naked. At ejaculation human sperm are reported to have as many as 300 different proteins on their surface, many of which are deposited after leaving the testes (Naaby-Hansen et al., 1997). It is reasonable to assume that the sperm surface is a complex structure, known to be divided into five separate anatomically distinct regions, each with a complement of functionally unique proteins (Cowan et al., 1997). Many of the components of the sperm surface glycocalyx are regionally localized and their association with the surface ranges from incorporation in the plasma membrane to loose adherence to the membrane associated proteins (Schroter et al., 1999). Currently, little is known about sperm coating proteins and their involvement in the capacitation process in primates. A number of factors associated with seminal plasma have been shown to inhibit aspects of capacitation in human sperm, although most have not been well-characterized nor has their mode of action been understood. It has been known for some time that whole seminal plasma contains factors that keep human

sperm in a non-capacitated state (Chang, 1957; Bedford, 1970; Begley and Quinn, 1982). Additions of dilute quantities of seminal plasma inhibit the development of hyperactivated motility associated with capacitation (Mortimer et al., 1998). A large molecular weight factor from human seminal plasma, termed anti-fertility factor-1 (AF-1), inhibits mouse sperm penetration into mouse oocytes (Audhya et al., 1987) as well as human sperm fusion with zona-free hamster oocytes (Van der Ven et al., 1982). Other factors found in the same semen fraction as AF-1 appear to inhibit the acrosome reaction (Han et al., 1990). This lack of a defined “decapacitation factor” is surprising considering the number of unique (glyco) proteins comprising the glycocalyx that have been identified in primate sperm (Schroter et al., 1999; for review). Yet the function of most of these proteins is still not understood, and it is possible that in time some of these surface components will be implicated in the process of capacitation.

The release of ESP13.2 and PSP94 following treatment with activator suggests that both coating proteins may be involved in some regulatory aspect of capacitation. ESP13.2 is unique in that it is localized to the entire sperm surface and not confined to a regional domain as seen with PSP94. A clear role for ESP13.2 in the capacitation process has been demonstrated in this study. Succinctly, ESP13.2 must be removed from the head of macaque sperm before specific sperm-zona pellucida binding can occur, and this release is concomitant with completion of capacitation. A clear role or function for PSP94 on sperm, however, has yet to be determined. The porcine homologue of PSP 94 has been shown to suppress the development of motility when added to sperm *in vitro* (Jeng et al., 1993). Subsequently called sperm motility inhibitor (SMI-1), this protein competitively inhibits the activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase in a dose dependent manner (Chao et al., 1996). Since Na<sup>+</sup>/K<sup>+</sup>-ATPase appears to be restricted to the flagellar membrane (Eddy EMaOB, 1992), it has been proposed that SMI-1 binds to and regulates the activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase on the sperm flagellum surface, although such a function of SMI-1 has yet to be demonstrated. In the present study, experimental conditions did not allow for direct assessment of the role that flagellum coating proteins may play in sperm capacitation. We were able to clearly show, perhaps for the first time in any species, that PSP94 resides on the sperm up to the final stages of capacitation and appears to be localized to the flagellum. It is interesting to note that sperm treated with caffeine or dbcAMP retained ESP13.2 and PSP94 over the tail, and they did not develop the hyperactivated movement characteristics associated with capacitation. Hyperactivated motility was only observed following treatment with both caffeine and dbcAMP, a condition which resulted in the complete release of ESP13.2 and PSP94 from the sperm flagellum. A role for both of these proteins in regulating hyperactivated motility remains a possibility. It is curious that these two surface proteins are still present until the end of capacitation, and that ESP13.2 is laid down in the terminal segment of the

epididymis, while PSP94 initiates association with the flagellum at the time of mixing with the seminal plasma. The conjecture that those proteins laid down first in the epididymis are the last to be removed in the female tract appears not to be true, at least in cynomolgus macaques.

Even though prolonged incubation in capacitation medium results in some loss of ESP13.2 from the sperm surface (Yudin et al., 2003), it is only when sperm are exposed to the pharmacologic activators dbcAMP and caffeine that ESP13.2 is extensively removed from the plasma membrane (Yudin et al., 2003). It has been recognized for some time that macaque sperm require both exogenous cAMP and caffeine for successful *in vitro* fertilization (Bavister et al., 1983; Wolf et al., 1989). The action of these compounds results in the synchronization of the final steps in the capacitation process (Tollner et al., 2003). The induction or enhancement of capacitation with addition of cAMP analogs in combination with methyl xanthines has also been reported for sperm of mouse, hamster, boar, bull, and human (Visconti et al., 1995, 1999; Kalab et al., 1998; Osheroff et al., 1999; Ho and Suarez, 2001a; Thundathil et al., 2002). The requirement for both compounds is not completely understood. It has been held that caffeine, a potent inhibitor of phosphodiesterases, acts by potentiating the effects of cAMP in sperm (Bavister et al., 1983; Boatman and Bavister, 1984). However, this mechanism does not appear to be involved in the loss of ESP13.2 following treatment with activators. Doubling the dose of dbcAMP alone has no effect on ESP13.2 release and increasing the dose of caffeine appears to release ESP13.2 only from the sperm head and not the flagellum.

The separate actions of caffeine and analogs of cAMP on sperm have been reported previously. Macaque sperm exposed to caffeine have been shown to initiate zona recognition and binding in 4 to 5 times greater numbers than sperm exposed to only dbcAMP or controls, while dbcAMP was most effective in promoting the acrosome reaction in sperm that had bound to the zona (VandeVoort et al., 1994). This earlier observation is consistent with the present finding that treatment with caffeine and not dbcAMP resulted in release of ESP13.2 from over the head, enabling sperm attachment to the zona. In humans, significant enhancement of sperm motility can be achieved after treatment with either caffeine or dbcAMP (Hong et al., 1985). The shape of the concentration-response curve and amplitude of motility increase with caffeine treatment was similar to that of EGTA, a calcium chelator, and lanthanum, a calcium blocker, while stimulation with dbcAMP was more similar to trifluoperazine, a calmodulin antagonist (Hong et al., 1985). Furthermore, the effect of caffeine, but not dbcAMP, was antagonized competitively by the addition of ionophore A23187 (Hong et al., 1985). In bovine sperm, caffeine restored motility and increased respiration in fluoride-immobilized sperm, while 8-bromo-cAMP (a highly permeable cAMP analog) did not. As in the case of human sperm, this effect of caffeine was not observed in bovine sperm that were permeabi-

lized (Schoff and Lardy, 1987). Caffeine also readily induced hyperactivated motility in bovine sperm, while the combination of dbcAMP and the methyl xanthine IBMX did not. This particular effect of caffeine was greatly reduced in the absence of extracellular calcium (Ho and Suarez, 2001b). Taken together, these studies suggest that caffeine works, at least in part, to modify calcium translocation in sperm.

The  $\text{Ca}^{++}$  mobilizing effects of caffeine have been described in sperm.  $\text{Ca}^{++}$  imaging of fluo-3FF/AM loaded bovine sperm showed a rapid rise in intracellular  $\text{Ca}^{++}$  in response to caffeine (Ho and Suarez, 2001b). In the same study, treatment with the same concentrations of caffeine were associated with rapid induction of hyperactivated motility that was not observed with addition of dbcAMP and IBMX. Similarly, boar sperm loaded with the  $\text{Ca}^{++}$  probe Fluo-3 also exhibited a caffeine-induced increase in intracellular  $\text{Ca}^{++}$ , a response that was not seen with additions of cAMP or activators of adenylate cyclase (Harrison et al., 1993). Theophylline, a methyl xanthine with a nearly identical structure to caffeine (Daly, 2000; for review) stimulated the uptake of  $\text{Ca}^{++}$  (isotope  $^{45}\text{Ca}^{++}$ ) in abalone sperm in a time and concentration dependent manner. The effect appeared independent of PDE inhibition as added cyclic nucleotides were without effect (Kopf et al., 1984).

The loss of ESP13.2 from over the sperm head and midpiece in response to caffeine may reflect a region specific release of  $\text{Ca}^{++}$  from intracellular stores. In somatic cell research, caffeine is one of the more potent methyl xanthines used for the activation of intracellular calcium store channels. The putative target of caffeine is a cyclic ADP ribose-modulated, intracellular ryanodine-sensitive calcium channel, resulting in release from intracellular stores (Herrmann-Frank et al., 1999; Daly, 2000; for review). Although there is no evidence as yet that these caffeine-sensitive channels exist in sperm, intracellular stores of  $\text{Ca}^{++}$  resolved with Fluo-3 have been identified in permeabilized human sperm and are limited to the acrosome and midpiece (De Blas et al., 2002). Furthermore, treatment of macaque sperm in our lab with thapsigargin, an inhibitor of  $\text{Ca}^{++}$  ATPase pumps associated with intracellular stores of  $\text{Ca}^{++}$  (Lytton et al., 1991), stimulated an identical pattern of ESP13.2 release over the sperm head and midpiece (unpublished data). This regional response to thapsigargin is consistent with the localization of fluorescence-conjugated thapsigargin to the identical regions in human sperm (Rossato et al., 2001).

In addition to regulating capacitation, ESP13.2 may also protect sperm from immunologic factors in the female reproductive tract. The presence of ESP13.2 along the entire length of the plasma membrane could mask key proteins from immunologic recognition. This possibility is suggested by the virtual absence of antibodies to sperm proteins (other than ESP13.2 and a 220 kDa protein) generated in rabbits when sperm were fixed to preserve surface proteins prior to immunization. Sperm injected following removal of ESP13.2 by activation, induced a rapid response to dozens of proteins. The

persistent binding of ESP13.2 to the sperm surface despite extensive washing procedures and overnight incubation suggests that tight association of ESP13.2 with the sperm surface is physiologically important, perhaps to provide protection during sperm ascent through cervical mucus, the uterine cavity and the upper reproductive tract. This possibility is supported by the observation that motile sperm collected from the uterus of mated female macaques are coated with ESP13.2 over the entire surface (unpublished data). The loss of ESP13.2, therefore, may not occur in vivo until sperm are close to the site of fertilization in the oviduct. Sperm that lose ESP13.2 prematurely in association with premature capacitation or cell death could stimulate phagocytosis by leukocytes that are known to migrate to the vagina, cervix, and uterus following insemination in mammals (Barratt and Pockley, 1998). This explanation sheds light on several observations of leukocyte response to sperm in the rabbit female tract. For example, leukocytes were observed to attach to motile rabbit sperm recovered from the uterus (Bedford, 1965). Also, there was no decrease in fertilization when female rabbits were mated with a second male following a previous mating that induced leukocyte influx into the reproductive tract (Taylor, 1982). If the proposed cloaking properties of ESP13.2 are correct, then loss of ESP13.2 from the sperm surface in vivo likely represents a very late step in capacitation, exposing receptors for the zona pellucida at a time when sperm are in proximity to the oocyte.

### CONCLUSIONS

In conclusion, we have shown that there are only two surface coating proteins that remain on the sperm surface through the in vitro capacitation process and are released upon addition of caffeine and cAMP. One of these glycoproteins, ESP13.2, is laid down during epididymal transit, while PSP94 becomes associated with the sperm at ejaculation and is a product of the prostate. ESP13.2 is shown to be released from over the head and midpiece of the sperm after treatment with caffeine. The action of caffeine does not appear to be mediated through cAMP, but may involve the stimulation of ESP13.2 release by increasing intracellular  $Ca^{++}$ . We have clearly demonstrated that the loss of ESP13.2 is critical for sperm recognition and binding to the zona pellucida. Our results also suggest that ESP13.2 may provide an immunoprotective shield, which could block sperm surface recognition by the female reproductive tract, thereby assuring a safe haven for sperm storage and ultimate capacitation.

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