

## Association of the Protein D and Protein E Forms of Rat CRISP1 with Epididymal Sperm<sup>1</sup>

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### ABSTRACT

Cysteine-rich secretory protein 1 (CRISP1) is a secretory glycoprotein produced by the rat epididymal epithelium in two forms, referred to as proteins D and E. CRISP1 has been implicated in sperm-egg fusion and has been shown to suppress capacitation in rat sperm. Several studies have suggested that CRISP1 associates transiently with the sperm surface, whereas others have shown that at least a portion of CRISP1 persists on the surface. In the present study, we demonstrate that protein D associates transiently with the sperm surface in a concentration-dependent manner, exhibiting saturable binding to both caput and cauda sperm in a concentration range that is consistent with its capacitation-inhibiting activity. In contrast, protein E persists on the sperm surface after all exogenous protein D has been dissociated. Comparison of caput and cauda sperm reveal that protein E becomes bound to the sperm in the cauda epididymidis. We show that protein E associates with caput sperm, which do not normally have it on their surfaces, *in vitro* in a time- and temperature-dependent manner. These studies demonstrate that most CRISP1 interacts with sperm transiently, possibly with a specific receptor on the sperm surface, consistent with its action in suppressing capacitation during epididymal storage of sperm. These studies also confirm a tightly bound population of protein E that could act in the female tract.

*acrosome reaction, CRISP1, epididymis, male reproductive tract, protein DE, sperm, sperm capacitation*

### INTRODUCTION

Cysteine Rich Secretory Proteins (CRISPs) are characterized by the presence of 16 highly conserved cysteine residues distributed among three domains; the CAP domain (also referred to as the pathogenesis-related or PR domain), the ion channel regulator (ICR) domain, and a hinge domain connecting the CAP and ICR domains. Together, the hinge and ICR domains have been referred to as the cysteine-rich domain (CRD) [1, 2]. These domains have been identified in the crystallized proteins, and each is stabilized by disulfide

links between the cysteines within the respective domains [2–5]. The CRISPs with demonstrated molecular activities have been shown to inhibit various types of ion channels [4–10].

In most mammals studied to date, there are three *Crisp* genes that are expressed in a number of organs. Human *CRISP1* and murine *Crisp4* appear to be orthologs and are expressed specifically in the epididymis [11–14]. *Crisp2* is expressed in testicular germ cells and becomes localized to the flagellum and the acrosomal matrix [15–19]. Human *CRISP3* and murine *Crisp1* appear to be orthologs and are expressed in the salivary glands, the male reproductive tract, and other tissues and cells [15–17, 20–23]. In the mouse, the *Crisp3* gene appears to be a duplication of *Crisp1* gene producing homologous gene products [13].

Rat CRISP1 has been shown to be produced in two forms referred to as proteins D and E [24]. These two forms of CRISP1, both products of the *Crisp1* gene, appear to differ by a single epitope [14]. We generated a monoclonal antibody named 4E9 that specifically recognizes the E-form of CRISP1 [25–27].

The aim of the present study was to further characterize the interaction of the D and E forms of CRISP1 with sperm. Herein we show that protein D only exists as a ~32-kDa protein that interacts transiently with the sperm surface and can be completely dissociated from the sperm easily by gentle washing. We confirm the presence of three molecular weight (MW) forms of protein E found in epididymal fluid and demonstrate that the intermediate form appears to interact transiently with sperm, whereas the small MW form becomes tightly bound and is not removed by simple washing. These results suggest that two populations of CRISP1 exist on the sperm surface and that they have distinct characteristics of association and, presumably, different functions.

### MATERIALS AND METHODS

#### *Chemicals and Reagents*

Horseshoe peroxidase-conjugated anti-phosphotyrosine monoclonal immunoglobulin G (IgG; 4G10) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Alexa Fluor 488 goat anti-rabbit IgG and SlowFade were purchased from Molecular Probes (Eugene, OR). Super Signal West Pico Chemiluminescent Substrate and BCA protein assay were purchased from Pierce Chemical Co. (Rockford, IL). Modified BWW (mBWW) was purchased from Irvine Scientific (Santa Ana, CA) or made in house. The SDS-PAGE gels and reagents were purchased from Bio-Rad (Hercules, CA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

#### *Antibodies*

The monoclonal antibody 4E9 used for detection of protein E has been described previously [25]. The epitope for monoclonal antibody 4C6 used for detecting proteins D and E was determined to be in the carboxyl-terminus of CRISP1 based on immunoreactivity with a recombinant protein produced from

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a cDNA clone encoding amino acids 182–246 of CRISP1 (data not shown). The 4C6 antibody exhibits identical staining patterns to monoclonal antibody 11D4, an antibody that we have previously characterized and which is directed against an epitope in the same region of CRISP1 [28]. Antibody E7 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) is an antibody against tubulin, a sperm flagellum protein, and was used as a loading and contamination control. All second antibodies were purchased from Sigma-Aldrich.

### *Isolation of Epididymal Sperm and Fluid and Testicular Sperm*

Male Spague-Dawley retired breeder rats were killed by CO<sub>2</sub> asphyxiation, and epididymes were surgically removed. The caput and/or cauda epididymes (depending on the experiment) were removed, cut several times with small surgical scissors, and incubated for 5 min in 1 ml mBWW (91 mM NaCl, 4.8 mM KCl, 2.4 mM MgSO<sub>4</sub>, 1.2 mM KPO<sub>4</sub>, 4 mM NaCO<sub>3</sub>, 21 mM HEPES, 5.6 mM glucose, 0.25 mM Na pyruvate, 1.7 mM Ca lactate, 21.6 mM Na lactate, and 5 µg/ml Phenol red, pH 7.4) at 37°C with gentle shaking. The medium containing sperm was placed in a 1.5-ml microcentrifuge tube and gently shaken to evenly distribute the sperm. Testicular germ cells were collected essentially as described by Nolan et al. [13]. Briefly, testis tissue was minced and cells dispersed by gentle rocking in mBWW for 15 min at 4°C. Dispersed cells were washed three times with cold mBWW by centrifugation at 500 × *g* for 1 min. Sperm were counted using a hemacytometer. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Epididymal fluid was obtained from the caput or cauda epididymes by placing the epididymal tissue in PBS (150 mM NaCl, 3 mM KCl, 1 mM KPO<sub>4</sub>, and 6 mM NaPO<sub>4</sub>) containing protease inhibitor cocktail and gently mincing the tissue with fine scissors. The resulting suspensions were centrifuged at 600 × *g* to remove tissues fragments. The supernatants were then recentrifuged at 14000 × *g* to remove the sperm cells. The supernatants were recovered and protein concentration determined by BCA protein assay.

### *Purification of CRISP1*

CRISP1 was purified from epididymal homogenates according to the protocol of Tubbs et al. [29], with slight modifications. Briefly, epididymes were homogenized in cold PBS containing protease inhibitor cocktail. Following centrifugation to remove cells and tissues, supernatant was filtered through Whatman no. 1 filter paper. The resulting filtrate was applied to a Con-A Sepharose column and washed with 20 mM phosphate buffer. Bound proteins were eluted with 0.2 M α-D-methyl mannoside. Fractions containing the protein peak were concentrated and buffer exchanged with 20 mM phosphate buffer containing 150 mM NaCl and applied to a Sephacryl-200 column. Resulting fractions were assessed for the presence of CRISP1 by Western blot analysis with anti-CRISP1 antibody. The fractions containing CRISP1 were combined and applied to a DEAE anion exchange column and eluted from the column with a 150–300 mM NaCl gradient. The CRISP1-containing fractions were determined by Western blot analysis, combined, and the purity assessed by silver stain following SDS-PAGE.

### *Western Blot Analysis*

Samples were subjected to PAGE on Tris-glycine gels under reducing denaturing conditions, and the proteins were subsequently transferred to Immobilon P membrane (Millipore, Bedford, MA) as previously described [30]. Immunodetection of the protein D and E forms of CRISP1 was done as previously described [28, 30].

### *Immunocytochemistry*

Sperm cells were treated in one of the following three ways: unwashed, one wash with mBWW by centrifugation at 600 × *g* (fast wash), or three washes with mBWW with two 15-min incubations between washes (dissociation wash). Following treatments, all cells were fixed with Bouin fixative for 45 min at room temperature. The fixed sperm were washed with 1 × PBS five times with centrifugation at 1300 × *g* for 5 min. After the final wash, sperm cells were incubated in a primary antibody (monoclonal antibody 4E9, or monoclonal antibody 4C6) in 1% BSA/PBS for 1 h. After the incubation, sperm cells were washed three times in PBS, and secondary antibody (anti-mouse IgG TRITC conjugate) in 1% BSA/PBS was added. Cells were incubated for 1 h at room temperature and then washed three times in PBS. To view, cells were mounted in SlowFade and observed with an Olympus fluorescent microscope.

### *Extraction of Sperm Membranes with Octyl-β-glucopyranoside*

Approximately 100 million sperm cells were extracted per 1 ml of 80 mM octyl-β-glucopyranoside (OBG). Cells were incubated in OBG at room temperature for 1 h while shaking. After the incubation, extracts were centrifuged at 16000 × *g* for 5 min, and the supernatants were recovered. Protein concentrations were determined using the BCA assay.

### *Acrosome Reaction*

The acrosome reaction was assessed as described previously [30]. Briefly, following capacitation incubation the acrosome reaction was induced by addition of progesterone (1 µM final concentration). Sperm were fixed in 4% paraformaldehyde, and the acrosome was visualized by staining with Coomassie blue G-250. Acrosomal staining was observed with bright-field microscopy (600× total magnification). A minimum of 500 cells were assessed for acrosomal status.

### *Equilibrium Binding of CRISP1 to Sperm*

Sperm were isolated from the caput and cauda epididymidis as described above and incubated at 37°C in the collection medium (dilute epididymal fluid) for 0, 1, or 3 h. Some aliquots were washed with mBWW at time 0 or 1 h, and were collected either immediately or after further incubation in mBWW. For Western blot analysis of bound forms of CRISP1, the sperm were centrifuged, as described above, and the pellet was dissolved directly in Laemmli sample buffer.

### *Saturation Binding Assay for the D Form of CRISP1*

Sperm were collected from the caput and cauda epididymidis as described above. After removing tissue fragments, sperm suspensions were incubated for 1 h at 37°C. Aliquots of 10 million sperm were then placed in tubes and centrifuged at 1000 × *g* for 5 min. The supernatants were removed, and sperm were washed in mBWW. Following removal of the wash, increasing amounts of CRISP1 in mBWW were added to the tubes. The tubes were incubated at 37°C for 2 h. Following centrifugation as above, the supernatants were removed, and sample buffer was added to each sperm pellet and prepared for Western blot analysis. The resulting films were scanned and data quantified using the Un-Scan-It program (Silk Scientific, Orem, UT).

### *Saturation Binding and Reversibility of the Protein E Form of CRISP1*

Proximal caput epididymal sperm and cauda epididymal fluid were obtained as described above. Cauda epididymal sperm were also collected to be used as a saturation control. Equal aliquots of proximal caput sperm were added to tubes and incubated at 33°C with cauda fluid (~8 mg/ml protein) for 5 min, 30 min, 2 h, or 18 h. At the designated times, tubes of sperm were washed with PBS to remove the cauda fluid, and 1 × 10<sup>5</sup> sperm were added to each well of an ELISA plate (Nunc Maxisorp; Nalgene Nunc International, Rochester, NY) that had been pretreated with poly-L-lysine (10 µg/ml). The sperm plates were centrifuged, extra fluid was removed, and the plate was placed in a vacuum dessicator. The ELISA with monoclonal antibody 4E9 was performed as previously described [25].

Additional tubes of proximal caput sperm and cauda epididymal fluid were prepared for each time point to be used for immunocytochemical analysis with monoclonal antibody 4E9 as described above.

To assess reversibility, proximal caput sperm were incubated with cauda fluid as described above for 5 min or 2 h, washed with PBS, and placed at 33°C in PBS for an additional 18 h. Sperm were adsorbed to the plates, and an ELISA was performed as described above.

### *Time and Temperature Binding Assay for Protein E Form*

Proximal caput sperm and cauda epididymal fluid were collected as described above. Proximal caput sperm were incubated with cauda fluid at 33°C for 5, 30, and 120 min. Controls included incubations with cauda fluid at 4°C and incubations with heat-denatured cauda fluid or PBS alone. After the incubations, sperm were washed twice with PBS and counted to determine cell number. For each condition, 1 × 10<sup>5</sup> sperm were added to wells of an ELISA plate, and the assay was performed as previously described [25].

### *Coculture of Sperm with Epididymal Epithelia*

Epididymes were removed from Sprague-Dawley retired breeder males and divided into five regions: proximal caput, distal caput, corpus, proximal cauda,

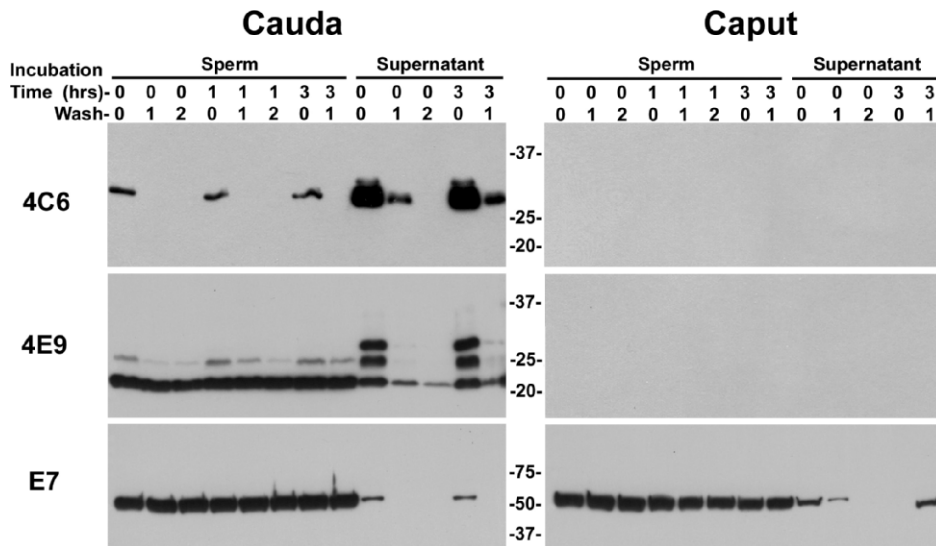


FIG. 1. The binding of CRISP1 forms to sperm as determined by Western blot analysis with antibodies to the far carboxyl terminus (4C6) and specifically to the protein E form of CRISP1 (4E9). After swimming out of the caput or cauda epididymidis, sperm were incubated with the dilute epididymal fluid in which they swam out. The sperm were washed at the times indicated and further incubated for the times indicated. At the end of the incubation, total sperm proteins (sperm) or an aliquot of the wash (supernatant) were analyzed by Western blotting. Each blot was stripped and reprobbed with all antibodies. The transient nature of binding by the large molecular weight form of CRISP1 (protein D) is apparent. The smallest molecular weight form of protein E detected by 4E9 cannot be removed from sperm by washing. There is no CRISP1 in caput fluid or bound to caput sperm. E7 is an antibody against rat tubulin and was included here as a control for the presence of sperm and as a loading control in the sperm lanes.

and distal cauda. Each region was placed in a separate tube in sterile HBSS (137 mM NaCl, 5.4 mM KCl, 0.25 mM  $\text{Na}_2\text{HPO}_4$ , 0.44 mM  $\text{KH}_2\text{PO}_4$ , 1.3 mM  $\text{CaCl}_2$ , 1.0 mM  $\text{MgSO}_4$ , and 4.2 mM  $\text{NaHCO}_3$ ) with protease inhibitor cocktail. Collagenase type IV (Sigma) was added to each tube to a final concentration of 10 mg/ml, and tubes were incubated in a gently shaking water bath at 37°C for 90 min. Resulting tubule fragments were washed four times with HBSS, allowing the fragments to pellet by gravity sedimentation. Culture medium consisting of Dulbecco modified Eagle medium (DMEM)/F12, 5% FBS, 20 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 250 nM testosterone was added to each tube. A sterile pipette was used to break the tubules into very small fragments and single cells. Aliquots of the resulting cell suspension, enriched in epithelial cells, were plated into wells of a 12-well tissue culture plate that had been coated with rat tail collagen I (Biocoat; BD Biosciences). The cells were allowed to recover and attach at 37°C, 5%  $\text{CO}_2$ , overnight. The following day, sperm were isolated from the caput epididymidis

by gently mincing freshly isolated proximal caput epididymal tissue in DMEM/F12 and allowing the sperm to disperse into the media. Sperm suspensions were collected and centrifuged at  $300 \times g$  for 5 min. The resulting sperm pellet was resuspended in culture medium at a concentration of  $2 \times 10^6$  cells/ml. Noncoated well inserts, 0.45- $\mu\text{m}$  pore size (Biocoat) were placed in the wells containing the epididymal epithelial explants, and 0.5 ml of the proximal caput sperm suspension ( $2 \times 10^6$  cells) was added to each insert. As controls, proximal caput sperm and distal cauda sperm were added to wells containing culture medium only. The plates were returned to the incubator for 3 days of coculture. After this time, sperm were harvested from the insert, washed, and immunostained as described above. The media from each experiment were subjected to SDS-PAGE and Western blot analysis.

## RESULTS

Characterization of CRISP1 sperm binding was investigated by Western blot analysis using antibodies specific to different forms of CRISP1. The 4C6 is a monoclonal antibody that is predicted to bind both the protein D and E forms of CRISP1 at an epitope located in the far carboxyl terminus of the proteins. The 4E9 is a monoclonal antibody specific for the protein E form of CRISP1 [25]. When sperm are eluted from the epididymis and total sperm proteins are extracted prior to washing, 4C6 detects a form of CRISP1 at 32 kDa on cauda rat sperm (Fig. 1). This form of CRISP1 remains in an equilibrium with unbound CRISP1 in the medium for at least 3 h (the longest time point tested), and the majority is removed from the surface of the sperm by a single washing. No protein is detected by 4C6 on caput sperm. These results show that this form of CRISP1 maintains a dynamic equilibrium through reversible binding to the to the sperm surface.

When monoclonal antibody 4E9 was used to probe these Western blots, a small-molecular weight form of CRISP1 was detected on cauda sperm (Fig. 1). This form of CRISP1 is consistent with the small-molecular weight form of protein E that we have reported previously as associated with cauda sperm [28]. This form of CRISP1 is tightly bound to cauda sperm, but it is not detected at all in caput sperm, and it is not recognized by the 4C6 antibody directed against the carboxyl terminus of full-length CRISP1.

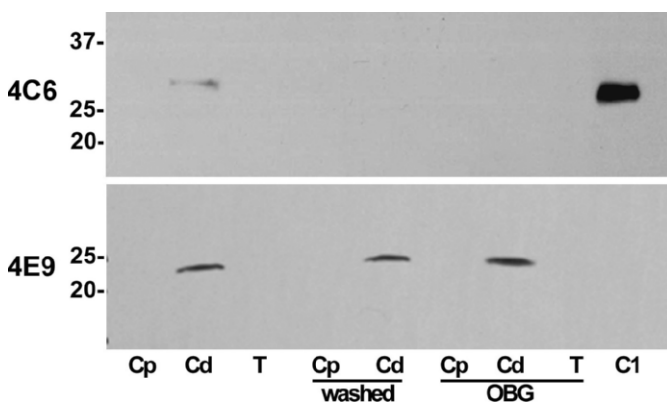


FIG. 2. To further characterize the binding of CRISP1 forms to rat sperm, Western blot analysis was carried out on whole-cell samples and detergent extracts (OBG) of epididymal sperm from the caput (Cp) or cauda (Cd), or of cells from the testis (T). Again, the large molecular weight form of CRISP1 is easily removed by washing, and the small molecular weight form of protein E is not. However, the E form of CRISP1 can be removed with detergent. Purified CRISP1 is included as a control (C1).

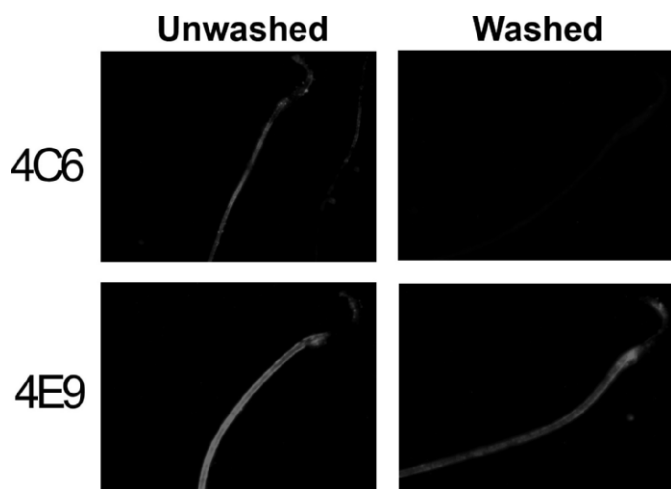


FIG. 3. Immunocytochemical analysis of unwashed and washed sperm from the caput or cauda epididymis. CRISP1 staining is observed with 4C6 only in unwashed epididymal sperm. The 4E9 staining appears the same for washed and unwashed cauda sperm, confirming the tight binding of protein E. No protein E is detected on caput sperm or testicular sperm. Original magnification  $\times 600$ .

To further characterize the origin and binding of these CRISP1 forms to sperm, a total population of testicular cells was isolated according to the method of Nolan et al. [13], in addition to caput and cauda sperm. Total proteins and the population of proteins extracted by OBG after washing were analyzed by Western blot analysis (Fig. 2). As expected, monoclonal antibody 4C6 recognizes only the D form of CRISP1 at 32 kDa. Monoclonal antibody 4E9 detected a 22-kDa band only on cauda epididymal sperm, and this protein was found in the detergent (OBG)-extracted fraction. These results show that proteins D and E interact with sperm in fundamentally different ways.

Immunocytochemical experiments were performed to confirm the observations of CRISP1 binding seen in the Western blots. Sperm were stained prior to washing or after washing to dissociate all reversibly bound CRISP1. Sperm were fixed immediately prior to immunocytochemistry to prevent loss of CRISP1 during the staining procedure. The results show 4C6 brightly stains the unwashed sperm, but when sperm are

washed the staining is completely diminished, consistent with the Western blot data (Fig. 3). As we have demonstrated previously, monoclonal antibody 4E9 shows strong binding to its antigen on the sperm tail, and this binding is unchanged by washing [25, 28]. These results support the Western blot data and support the idea that the full-size D form of CRISP1 is completely lost from the sperm by washing, but that the E form remains tightly associated with the sperm surface.

We have shown previously that purified CRISP1, which is more than 90% composed of the D form of the protein, is able to inhibit capacitation of sperm with maximal inhibitory activity at 400  $\mu\text{g/ml}$  [30]. When the CRISP1 is removed, capacitation resumes, consistent with the reversible interaction of the full-length form of CRISP1 with sperm. To determine whether the reversible interaction that we have demonstrated here is consistent with a saturable binding, we evaluated the amount of CRISP1 bound to sperm with increasing CRISP1 concentration. Figure 4A shows that CRISP1 binding to caput or cauda epididymal sperm reaches a maximum at a concentration of 400  $\mu\text{g/ml}$  pure CRISP1. To confirm that this range of binding activity conforms to the capacitation inhibitory activity, we performed a dose-response study for capacitation inhibition by pure CRISP1 using progesterone-induced acrosome reaction as the endpoint. Figure 4B shows the results of this dose-response study. Maximum CRISP1 inhibition of capacitation was reached at 200  $\mu\text{g/ml}$ , and partial inhibition was still evident at 50  $\mu\text{g/ml}$ . These data are consistent with the binding data of Figure 4A and suggest that CRISP1 binds in a saturable manner to the sperm surface and inhibits capacitation as a result. We further show that the binding of CRISP1 to caput sperm is essentially the same as to cauda sperm, suggesting that the binding site for the D form of CRISP1 may be present on sperm as they leave the testis (Fig. 4A).

In contrast to the reversible binding of the full-size D form of CRISP1 to sperm, the binding of the E form of CRISP1 to sperm is extremely tight and can only be removed by detergent (Figs. 1 and 2). Initial attempts to load caput epididymal sperm with purified protein E were unsuccessful. However, protein E can be loaded onto the sperm surface when caput sperm are incubated in cauda epididymal fluid (Fig. 5A). In this experiment, caput sperm devoid of protein E were incubated with cauda epididymal fluid for increasing amounts of time. At

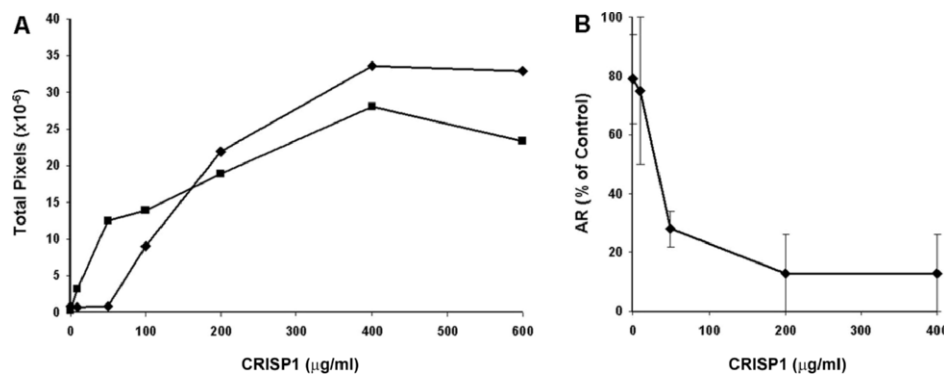
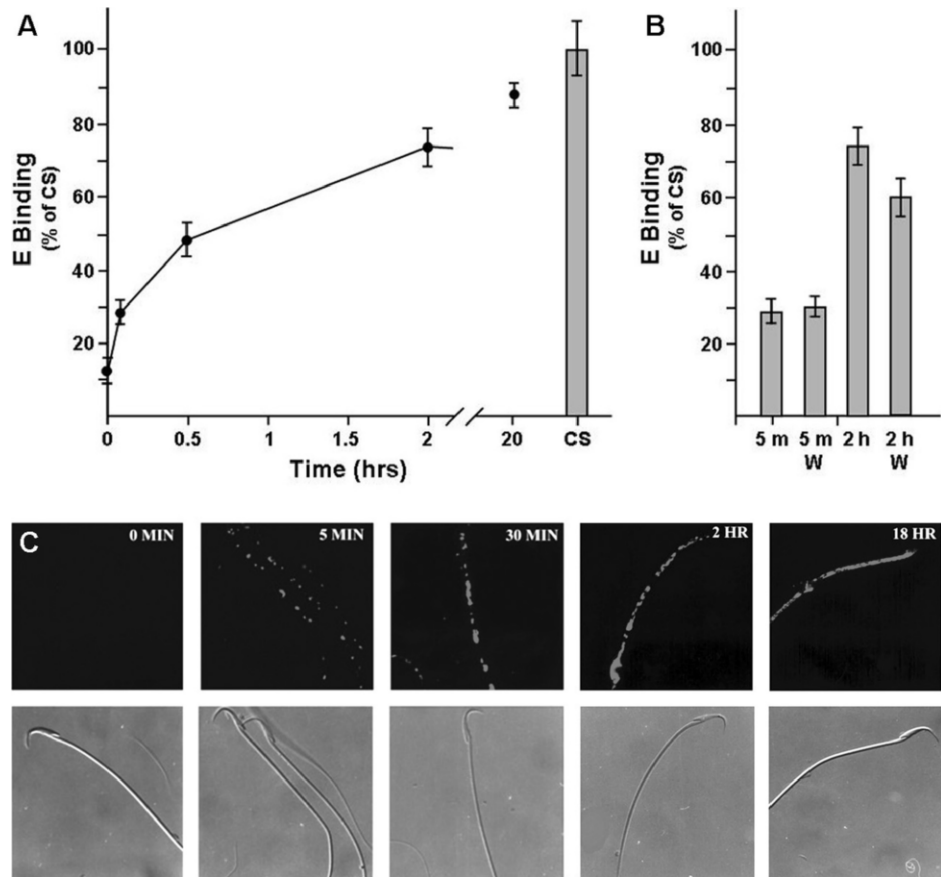


FIG. 4. **A**) Saturation binding of CRISP1 to caput (squares) and cauda (diamonds) sperm. Sperm were incubated in increasing concentrations of purified CRISP1. Following washing to remove unbound CRISP1, total sperm proteins were subjected to Western blot analysis for detection of CRISP1. Relative amounts of bound CRISP1 were determined semiquantitatively by densitometric scanning of the Western blot films. Saturation of CRISP1 binding to the sperm occurs at a concentration of 400  $\mu\text{g/ml}$ . **B**) Dose-dependent inhibition of capacitation of rat sperm as determined by progesterone-inducible acrosome reaction. Cauda sperm were capacitated in the presence of increasing concentrations of purified CRISP1, followed by induction of the acrosome reaction with progesterone. Acrosome-reacted sperm (AR) were determined by staining with Coomassie Blue. The data are presented as percentage of control (sperm incubated under noncapacitating conditions) and clearly show a complete inhibition of capacitation at 200  $\mu\text{g/ml}$  CRISP1, consistent with the dose range of CRISP1 saturation binding to the sperm. Errors bars indicate SEM.

FIG. 5. Characteristics of binding of the protein E form of CRISP1 to rat sperm. **A**) Aliquots of proximal caput epididymal sperm were incubated with epididymal fluid at 33°C for increasing amounts of time. At the designated times (5 min, 30 min, 2 h, or 18 h), aliquots of sperm were washed, and the sperm-bound protein E was determined by ELISA with monoclonal antibody 4E9. Protein E binds to sperm *in vitro* to nearly the same saturation as that found *in vivo*, as illustrated by sperm isolated from the cauda epididymidis (CS) that served as a control. **B**) The strength of the interaction between protein E and sperm was determined by washing (W) proximal caput sperm that had been preloaded in epididymal fluid for 5 min or 2 h. Following a wash incubation of 18 h in PBS, the bound protein E was determined by ELISA with antibody 4E9. The protein E that binds *in vitro* is as tightly bound as that loaded *in vivo*. Error bars indicate SEM. **C**) The loading of protein E determined by ELISA was confirmed visually by immunocytochemistry using antibody 4E9. Original magnification  $\times 600$ .



each time point, the sperm were washed and the amount of protein E associated with the sperm was determined by ELISA assay. This experiment showed a time-dependent association of the E form of CRISP1 with the sperm, which reached a near maximum by 2 h. Maximal binding was achieved by 20 h and was nearly the same as native cauda sperm. This loading of protein E was confirmed visually by immunocytochemistry

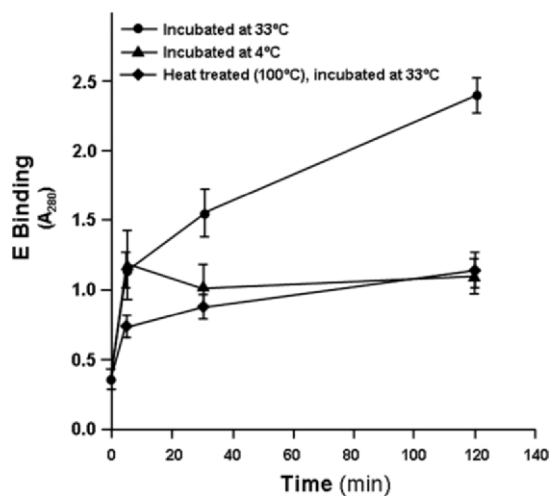


FIG. 6. Temperature dependence of protein E binding to sperm. Proximal caput sperm were incubated in epididymal fluid at 33°C or 4°C, or at 33°C in epididymal fluid that had been preheated to 100°C. Bound protein E was determined by ELISA with monoclonal antibody 4E9. Protein E binds to sperm at both 33°C and 4°C, but loading only occurs at the physiological temperature of 33°C. Heating the epididymal fluid destroys binding activity. Error bars indicate SEM.

(Fig. 5C). As with the protein E that is found on cauda sperm, the protein E that was loaded onto the caput sperm *in vitro* was bound tightly and was unable to be removed by washing (Fig. 5B). This is evidence that the *in vitro* binding reflects the *in vivo* binding of protein E.

The fact that the loading of protein E could be accomplished with epididymal fluid suggested that the process was specific and active. To test this hypothesis, caput sperm were loaded with epididymal fluid at 4°C or in epididymal fluid that had been previously heated to 100°C to destroy active proteins in the fluid. Both of these experimental manipulations dramatically decreased protein E binding to the sperm compared with control, suggesting that the binding of protein E to the sperm is specific and requires native protein (Fig. 6).

To determine whether the factors required for protein E binding to sperm were produced by the epididymal epithelium and whether the loading of sperm with the E form requires direct interaction with the epididymal epithelial cells, caput sperm were incubated above cultured cauda epididymal cells, separated from those cells by a 0.45- $\mu$ m filter. Protein E binding was determined nonquantitatively by immunocytochemistry. The results clearly show that cultured cauda epididymal cells produce the same forms of protein E found in epididymal fluid (Fig. 7A), as well as the other factors required to load protein E onto the sperm surface (Fig. 7B).

## DISCUSSION

We have shown previously that the predominant form of CRISP1, the protein D form, appears to interact reversibly with the sperm surface [30, 31]. Here, we further this characterization by showing that protein D maintains equilibrium binding with the sperm surface and that this binding is saturable at a

CRISP1 concentration of 400  $\mu\text{g/ml}$  (12.5  $\mu\text{M}$ , assuming a molecular mass of 32 kDa). This affinity of CRISP1 for the sperm surface is low compared with that seen for the activity of venom Crisp proteins, which have been shown to interact with ion channels in the plasma membrane at inhibitory concentrations in the nanomolar range [4, 6, 10]. Nevertheless, CRISP1 inhibits capacitation in the same concentration range as the saturable binding to sperm, suggesting that the binding data are relevant to this physiological activity of CRISP1. We have determined the concentration of CRISP1 in epididymal fluid by ELISA assay to be 1.6 mg/ml (50  $\mu\text{M}$ ), well in excess of a saturating dose of 12.5  $\mu\text{M}$  and consistent with a physiological effect of suppressing capacitation during sperm storage in the cauda epididymis. The concentration of CRISP1 required to suppress sperm-egg fusion is similar (200  $\mu\text{g/ml}$  or 6  $\mu\text{M}$ ), suggesting that CRISP1 may block sperm-egg fusion by a molecular mechanism similar to that for the inhibition of capacitation [32–34].

Although consistent with the demonstrated activity of the protein D form of CRISP1, other binding studies using iodinated CRISP1, or proteins presumed to be CRISP1, show much higher binding affinities (low nanomolar range) for at least a population of the protein [35, 36]. In our attempts to label/purify CRISP1, all levels of iodination eliminated the binding of the protein to the sperm surface. CRISP1 is a tyrosine-rich protein, and iodine is a huge molecular adduct. It is not hard to conceive of how low-affinity binding would be lost by modification of even a few key tyrosine residues. What is not clear is why the binding data presented here are in apparent conflict with these previous studies, especially since we use the same CRISP1 purification protocol published by Tubbs et al. [29].

The mechanism by which CRISP1 acts to suppress capacitation is unknown. Given the structural relationship of mammalian CRISP1 to toxin molecules of the Crisp family, it is possible that it has a similar mechanism of action. The Crisp toxins studied to date act by blocking ion channels, including ryanodine receptors, cyclic nucleotide gated (CNG) channels, and potassium channels [4–7, 10, 37]. These toxins act on their target channels *in vitro* in a concentration range of 0.1 to 1  $\mu\text{M}$ . If CRISP1 affects rat sperm capacitation by blocking an ion channel, the effective concentration for this inhibition is at least an order of magnitude higher than that found for venom toxin Crisp proteins.

In most mammalian species, including the rat, the relatively small contribution of epididymal fluid to the total volume of the ejaculate precludes the possibility that epididymal proteins, other than those that become bound to sperm, could have important functions in the female tract. This may not be the case for the protein D form of CRISP1. Even at a 100-fold dilution into the total ejaculate, the concentration of this protein would be in the high nanomolar concentration range. As discussed above, this is a concentration range in which venom Crisp proteins have been shown to have activity, and it is therefore possible that CRISP1 has an activity at this concentration in the female tract.

In contrast to protein D, the protein E form of CRISP1, which is specifically recognized by our 4E9 monoclonal antibody, binds in an essentially irreversible manner to the sperm surface. *In vitro* binding experiments can mimic the irreversible nature of protein E binding to sperm and also show that binding appears to require components produced by the epididymal epithelia. Protein E is produced by the epididymal epithelium in three distinct MW forms. The highest of these forms is the same size as protein D. The reason for the apparent decrease in size of the other forms is not known, but we have

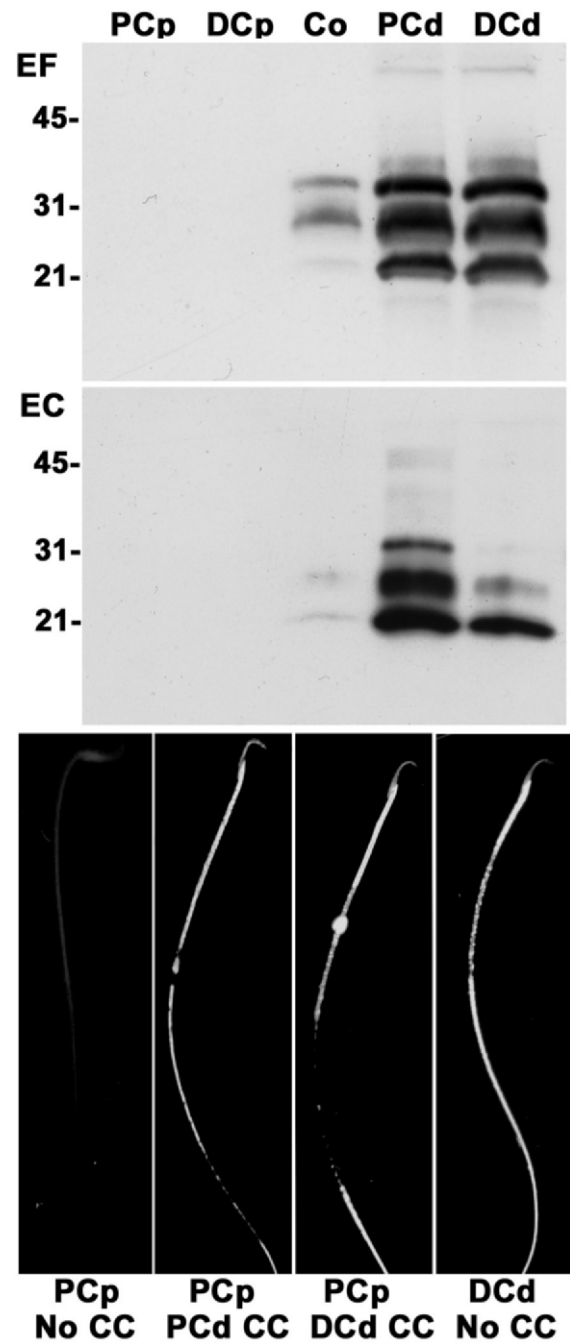


FIG. 7. Forms of protein E, as determined by Western blot, produced by region-specific cultured epididymal epithelial cells (EC; middle panel) compared with forms produced regionally by the epididymis *in vivo* (EF; upper panel). The regions of the epididymis used are proximal caput (PCp), distal caput (DCp), corpus (Co), proximal cauda (PCd), and distal cauda (DCd). The three forms of protein E are produced primarily by the cauda epididymal epithelium, and this differentiation is maintained in short-term culture. When proximal caput sperm are cocultured with cauda epithelial cells (CC), protein E loads on these sperm in a manner indistinguishable from the loading observed using epididymal fluid (Fig. 5) and mimics the binding found to distal cauda sperm (lower panel). Original magnification  $\times 600$ .

some evidence based on epitope mapping that this may represent proteolytic processing of protein E [28, 30, 31]. This contention remains to be definitively established experimentally. It is clear that only the smallest MW form of protein E becomes bound to the sperm surface. The function of this form of protein E is not known, but since it does not dissociate from

the sperm, it is possible that it has a function in the female tract. It is of interest to note that our antibodies do not detect any protein D remaining permanently bound to the sperm head, in contrast to results shown by others [32, 38]. The reason for this discrepancy is not clear.

The loading of protein D onto caput sperm shows essentially the same saturability in the same concentration range as that seen for cauda sperm. This suggests that whatever the binding partner for protein D is on the sperm surface, it is present in caput sperm and may be a germ cell product expressed in the testis. The same is true for the loading of protein E. Although the nature of the binding is completely different from that of protein D, the binding site on sperm is present in caput sperm.

Our lab has established a role for CRISP1, particularly the protein D form, in the suppression of capacitation. As such, it can be grouped with other decapacitation factors that have been characterized [39, 40]. We hypothesize that the activity of the D form of CRISP1 would function to stabilize sperm during storage in the cauda epididymis. Loss of CRISP1 may be expected to result in premature capacitation of sperm during storage in the epididymis and, consequently, a reduction in fertility. A knockout mouse targeting the *Crispl* gene has recently been reported [41]. Sperm from these mice show reduced fertilizing ability in vitro, but the male mice are fertile. Sperm from these mice show aberrant tyrosine phosphorylation when incubated under capacitating conditions but undergo a progesterone-induced acrosome reaction similar to normal sperm [41]. These results are not inconsistent with a capacitation-suppressive function for CRISP1. Further experiments focused on the timing of capacitation events in sperm from *Crispl* knockout mice could shed light on the potential role of CRISP1 in epididymal sperm storage.

In summary, we have shown that the high-molecular weight form of CRISP1 (protein D) that can be detected by monoclonal antibody 4C6 is readily dissociated from the surface of rat sperm. The binding affinity of this form of CRISP1 is consistent with the concentration range required for its role in inhibiting capacitation, and the high concentration of CRISP1 in epididymal fluid suggests that it functions to inhibit capacitation during epididymal storage of sperm. A smaller molecular weight form of CRISP1 that is detected with our protein E-specific antibody (4E9) becomes tightly bound to the sperm, and undoubtedly persists on sperm within the female tract. The tight binding of protein E requires other factors produced by the epididymal epithelium, in contrast to the reversible binding of Protein D.

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