MOLECULAR REPRODUCTION AND DEVELOPMENT 56:180–188 (2000)

Relationship Between the Association of Rat Epididymal Protein "DE" With Spermatozoa and the Behavior and Function of the Protein

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ABSTRACT Rat epididymal glycoprotein DE associates with the dorsal region of the sperm head during sperm maturation, migrates to the equatorial segment (ES) with the acrosome reaction (AR), and is involved in gamete membrane fusion. In the present study we examined the association of DE with the sperm surface and the relationship of this interaction with the behavior and function of the protein. Cloning and sequencing of DE revealed a lack of hydrophobic domains and the presence of 16 cysteine residues in the molecule. Experiments in which cauda epididymal sperm were subjected to different extraction procedures indicated that while most of the protein is removable from sperm by mild ionic strength, a low amount of DE, resistant to even 2 M NaCl, can be completely extracted by agents that remove integral proteins. However, the lack of hydrophobic domains in the molecule and the failure of DE to interact with liposomes, does not support a direct insertion of the protein into the lipid bilayer. These results, and the complete extraction of the tightly bound protein by dithiothreitol, suggest that this population would correspond to a peripheral protein bound to a membrane component by strong noncovalent interactions that involve disulfide bonds. While ELISA experiments showed that no protein could be extracted by NaCl from capacitated sperm, indirect immunofluorescence studies revealed the ability of the NaCl-resistant protein to migrate to the ES. Together, these results support the existence of two populations of DE: a major, loosely bound population that is released during capacitation, and a minor strongly bound population that remains after capacitation, migrates to the ES with the AR, and thus would correspond to the one with a role in gamete fusion. *Mol. Reprod. Dev. 56:180–188, 2000.* © 2000 Wiley-Liss, Inc.

Key Words: sperm membrane; maturation; capacitation; fertilization

INTRODUCTION

Transit through the epididymis confers functional maturity to sperm, evidenced by the progressive acquisition of the capacity for vigorous motility, the ability to become capacitated, undergo the acrosome reaction, interact with the zona pellucida, and bind to and fuse with the egg plasma membrane (for reviews see Cooper, 1986; Bedford, 1990; Yanagimachi, 1994). Numerous reports have demonstrated that the association of androgen-dependent epididymal secretory proteins to the sperm surface during maturation plays an important role in the development of sperm fertilizing ability (Cuasnicú et al., 1984; Sanjurjo et al., 1990; Bérubé et al., 1996; Boué et al., 1996; Moore, 1996).

Rat epididymal protein DE, first described by our laboratory in 1976 (Cameo and Blaquier, 1976), is synthesized and secreted by the epithelium of the proximal segments of the epididymis in response to androgens. Indirect immunofluorescence (IIF) studies using a specific polyclonal antibody against DE (anti-DE) demonstrated that the protein associates with the sperm surface during epididymal transit and localizes on the dorsal region of the sperm head (Kohane et al., 1979; Rochwerger and Cuasnicú, 1992).

The fact that a substantial amount of DE can be released from the sperm surface with either mild ionic strength (NaCl 0.4 M) or incubation under capacitating conditions first suggested that DE might be acting as a decapacitating factor (Kohane et al., 1980b). Subsequent experiments revealed however, the permanence of DE after capacitation (Cameo et al., 1986) and its migration from the dorsal region to the equatorial segment of the sperm head concomitantly with the occurrence of the acrosome reaction (AR) (Rochwerger and Cuasnicú, 1992). The relocalization of DE to the equatorial segment, the region through which the sperm fuses with the egg plasma membrane (Bedford et al., 1979; Yanagimachi, 1988), suggested that the protein might play a role in this event of fertilization. Subsequent experiments in which the presence of DE during gamete co-incubation significantly inhibited the percentage of both penetrated zona-free eggs and eggs with fused sperm supported a role for DE in gamete-membrane fusion through complementary sites for the

Grant sponsor: World Health Organization; Grant number: H9/181/R429.

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Received 6 July 1999; Accepted 15 December 1999

protein on the egg surface (Rochwerger et al., 1992; Cohen et al., 1996).

While the removal of the protein by moderate ionic strength suggests that DE might be loosely associated with the sperm plasma membrane, the permanence of DE after capacitation and even after the AR, together with its role in fertilization, suggests that the association of DE with the sperm surface should be stronger than that expected for a protein bound to the sperm by ionic interaction.

In view of these observations, the aim of the present study was to examine the association of DE with the sperm surface and the relationship between this interaction and the behavior and function of the protein.

MATERIALS AND METHODS

Animals

Adult (90–120 days old) male Sprague-Dawley rats were maintained at 23°C with a 12-hour-light/12-hour-dark cycle. Experiments were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction.

Amino Acid Sequencing

Purified protein DE (Garberi et al., 1979, 1982) was separated in 10% polyacrylamide gels, stained with Coomassie blue, and the corresponding band carefully excised from the gel. The gel containing DE was then subjected to trypsin digestion, and the tryptic peptides were isolated by high pressure liquid chromatography (HPLC). After the selection of peptides for Laser Desorption Mass Spectrometry (LDMS), peptides were sequenced by Edman degradation following standard procedures (Biotechnology Resource Laboratory, W.M. Keck Foundation, Yale University, New Haven, CT).

Isolation of cDNA Clones Encoding DE

A rat epididymal cDNA library (constructed in λgt11) was plated on Y090 cells and incubated at 42°C until lysis plaques appeared. A nitrocellulose filter soaked in 10 mM isopropyl-β-galactopyranoside was then placed over the agar, and the dishes were incubated overnight at 37°C. Positive clones were detected by incubation of the nitrocellulose membranes with anti-DE antibody (Kohane et al., 1983; see procedures described for Western blot), and then cut out of the agar and subjected to secondary and tertiary screening. Automatic sequencing of both strands of the inserts was performed at the Instituto de Ingeniería Genética y Biología Molecular (Buenos Aires, Argentina), using commercial λgt11 sequencing primers (Promega Corporation, Madison, WI).

Sperm Extracts

Epididymal sperm were allowed to disperse in PBS at 37°C, washed three times in PBS containing 0.2 mM PMSF (PBS-PMSF), and then incubated for 30 min in 2M NaCl PBS. The suspension was centrifuged 10 min at 13,000*g*, and the supernatant dialysed against deionized water at 4°C and lyophilized. To examine the association of the tightly bound protein with the sperm surface, the cells were incubated with 2 M NaCl PBS for 30 min, the suspension was centrifuged at 700*g*, and the pellet was incubated once again with 2M NaCl, as described above. After centrifugation at 700*g*, the sperm were incubated for 30 min with either 1% Triton X-100 in PBS, 50 mM glycine (pH = 3), 100 mM Na_2CO_3 (pH = 11), 6 M guanidine hydrochloride, 8 M urea, 100 mM dithiothreitol (DTT), or PBS as a control. Following these incubations, suspensions were centrifuged for 10 min at 13000*g*. The Triton X-100 supernatant was precipitated at −20°C with 10 volumes of acetone. The remaining supernatants were dialysed against deionized water at 4°C and lyophilized.

For the collection of testicular cells, the seminiferous tubules were released in PBS by making a small incision in the caudal end of the testis, washed two times, and minced in 6 ml of PBS for 10 min with fine scissors. The suspension was pippeted for several times and then filtered through 80-μm mesh. The cells were pelleted by centrifuging at 700*g*, washed by resuspending in PBS, and subjected to extraction procedures described above. Testicular cytosol was prepared as previously described (Kohane et al., 1979).

Indirect Immunofluorescence

Sperm were fixed for 10 min in 2% paraformaldehyde in PBS at room temperature. After extensive washing with PBS, sperm were air-dried on poly-L-lysine (0.01 mg/ml)-coated slides, incubated with normal goat serum (NGS, 5% in PBS) for 30 min at 37°C, and then incubated with anti-DE (1:100 in NGS, 1% in PBS) overnight at 4°C. After washing, sperm were incubated for 30 min at 37°C in fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG (1:100 in PBS; Sigma, St. Louis, MO), washed, and mounted in 90% glycerol in PBS. More than 200 cells for each preparation were examined with a Nikon Optiphot microscope equipped with epifluorescent optics.

Western Blots

Sperm extracts were separated in nondenaturing, nonreducing 7.5% or 10% polyacrylamide gels, according to the method of Hames (1981), and proteins were electrotransferred to nitrocellulose (Towbin et al., 1979). The membranes were blocked for 1 hr with powdered skim milk (2% in PBS) and incubated 2 hr with anti-DE antibody (1:200 in blocking solution). Membranes were thoroughly washed before incubation for 1 hr with biotin-conjugated antirabbit IgG (1:500 dilution, Sigma). After extensive washing, the membranes were incubated for 1 hr with ExtrAvidin-horseradish-peroxidase (1:1000 dilution, Sigma), and reactive bands were visualized with 3,3′-diaminobenzidine (40 μg/ml in Tris 0.1 M, pH = 7.5, 0.01% H_2O_2). All incubations were carried out at room temperature.

Preparation of Liposomes and Binding Assays

Small unilamellar vesicles of phosphatidylcholine (PC) or PC:cholesterol (7:3) were prepared by sonication of 1 mg/ml dispersions of lipids (Avanti Polar Lipids Inc, Alabama) in an ice bath for 5 min. Binding assays were performed by incubating different concentrations of liposomes (0.1–1 mg), with or without protein DE (5 µg), in a total volume of 1 ml of either PBS or TL-HEPES (pH = 7.2; Bavister, 1989), without sodium lactate and glucose, at either room temperature or 37°C. After 1 hr, the samples were ultracentrifuged at 40,000*g* for 30 min at 4°C. The pellet containing the liposomes was resuspended in 350 µl of buffer, and the emission fluorescence spectra was determined in a spectrofluorometer (SLM AMINC, SLM Instruments Inc., Cambridge, UK) equipped with a thermostatted cell holder (excitation: 280 nm, emission range: 300–500 nm, T: 25°C, gain: 100 mV/mA, HV: 300–700 mV).

In Vitro Sperm Capacitation

The standard capacitation medium used was described by Kaplan and Kraicer (1978). Spermatozoa were recovered from the cauda epididymis by incising the larger-diameter tubules in 1 ml of capacitation medium, and motile sperm were selected by swim up (15 min at 37°C). Aliquots of the motile sperm suspension were diluted in fresh capacitating medium, and the final sperm concentration was adjusted to $0.5–1 \times 10^6$ cells/ml. Tissue culture wells (16 mm; Costar Corporation, Cambridge, MA) containing 500 µl of sperm suspension were incubated for 5 hr under paraffin oil (Fisher, Pittsburgh, PA; saybolt viscosity 125/135) at 37°C in an atmosphere of 5% CO_2 in air. For acrosome reaction induction, the sperm were incubated under capacitating conditions for 4 hr, and Ca^{2+} ionophore A23187 (Sigma) was added during the last hour (final concentration of 1 µM). To obtain the protein released during capacitation, capacitated sperm suspensions were centrifuged at 700*g* for 10 min, and the supernatant was stored at −20°C.

ELISA

Fifty microliters of either fresh epididymal sperm or sperm subjected to different extraction procedures (1×10^6 sperm/ml) were placed in each well of microtiter plates (Nunc A/S, Roskilde, Denmark). The plates were centrifuged for 10 min at 530*g* and then fixed for 10 min with 2% glutaraldehyde. After blocking nonspecific binding sites for 60 min with 20 mg/ml powdered skim milk in PBS, anti-DE (1:200 in blocking solution) was added to duplicate wells and then incubated for 90 min. The wells were then washed with PBS supplemented with 0.02% Tween 20 (PBS-T), and subsequently incubated 60 min with biotin-conjugated anti-rabbit IgG (1:500 in 0.1% BSA in PBS). After washing with PBS-T, ExtrAvidin-horseradish-alkaline phosphatase (Sigma; 1:1,000 in 0.1% BSA in PBS) was added to each well and incubated for 45 min. All incubations were carried out at room temperature. After washing with PBS-T, 1 mg/ml of p-nitro-phenyl-phosphate (Sigma) in diethanolamine buffer was added (10% diethanolamine, 0.01% $MgCl_2$, pH = 9.8), and the color reaction was allowed to develop. Absorbance at 405 nm was determined with a microplate reader (Cambridge Technology, Inc., Watertown, MA).

RESULTS

Cloning and Sequencing of DE

As a first approach towards the understanding of the association of DE with the sperm plasma membrane, the amino acid sequence of the purified protein was determined. Trypsin digestion of DE produced several peptides, five of which were chosen for LDMS. Two peaks were then selected for their suitability for sequencing, one of which (Mw 2156.1), could not be sequenced due to the existence of a blocked N-terminus. The second peptide yielded a 13-residue sequence that matched 100% with those reported for residues 65–77 in both the mature epididymal secretory glycoprotein sequenced by Brooks and colleagues (1986) and the acidic epididymal glycoprotein sequenced by Charest and colleagues (1988).

The amino acid sequence of DE was also determined by screening a λgt11 rat epididymal cDNA library with the polyclonal antibody anti-DE. One of the positive clones was sequenced and presented 100% identity, with the last 165 coding base pairs and the following 94 base pairs of the noncoding 3′ region of the sequence previously reported by Brooks and colleagues (1986) and Charest and colleagues (1988). The analysis of the complete sequence indicates that the molecule lacks hydrophobic domains and presents 16 cysteine residues, 10 of which are located in the carboxy-terminal region.

Interaction of DE With the Sperm Plasma Membrane

Although a substantial amount of DE could be removed from sperm by moderate ionic strength (NaCl 0.4 M), a remnant of DE could be detected on the sperm surface after this treatment (Kohane et al., 1980b). In order to examine whether higher concentrations of NaCl could remove all the protein from the sperm surface, cauda epididymal sperm were incubated with 2 M NaCl and then subjected to IIF. Results showed that, in spite of the high salt concentration used, 95% of the cells were still showing the typical fluorescence pattern on the dorsal region of the sperm head (Fig. 1a–d).

In order to examine the association of the NaCl-resistant population with the sperm surface, cauda epididymal sperm which had been previously treated with 2 M NaCl were exposed to different treatments, which included low (3) and high (11) pH conditions, guanidine hydrochloride, urea, and Triton X-100. The presence of DE in the protein extracts and on the sperm surface was subsequently analyzed by Western blot and IIF, respectively. While low pH was not capable of removing DE from the sperm, the protein could be

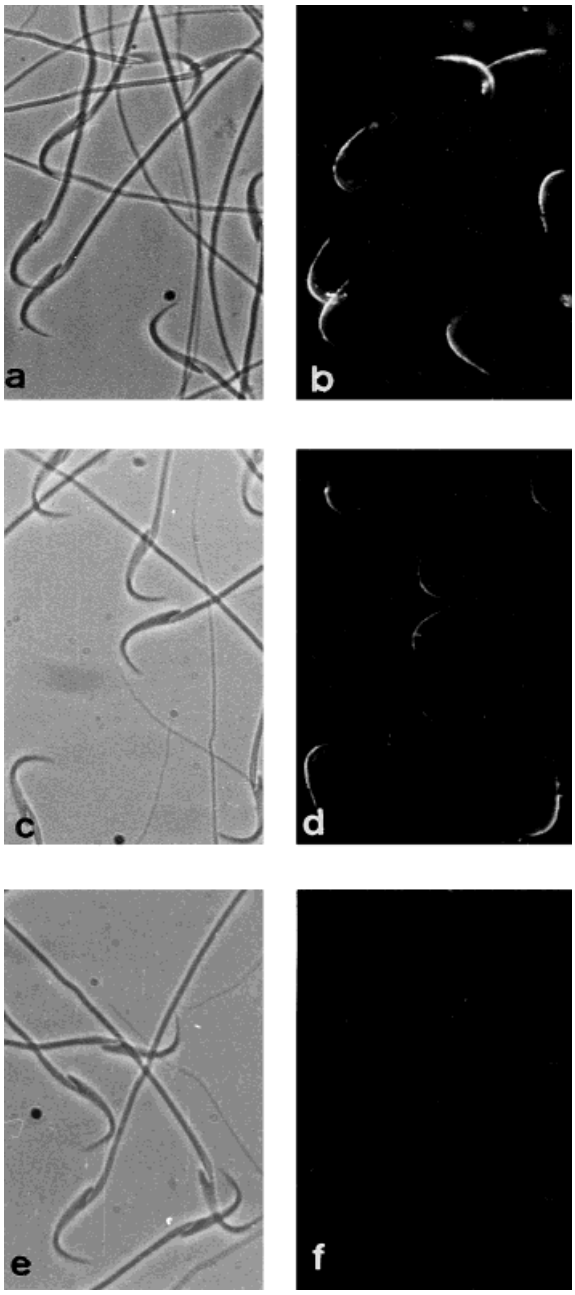

Fig. 1. Phase contrast (left) and immunofluorescence (right) of sperm treated with 2 M NaCl. **a, b:** Fresh cauda epididymal sperm were fixed and subjected to IIF. **c, d:** Sperm were treated with 2 M NaCl, fixed, and subjected to IIF. Note the presence of fluorescent labeling on the dorsal region of the sperm head. **e, f:** Sperm were exposed to 2 M NaCl, incubated with 1% Triton X-100, fixed, and subjected to IIF. Note the absence of fluorescence on the cells. Identical results were observed when the sperm were exposed to 100 mM Na_2CO_3, pH = 11, 6 M guanidine hydrochloride, or 100 mM DTT.

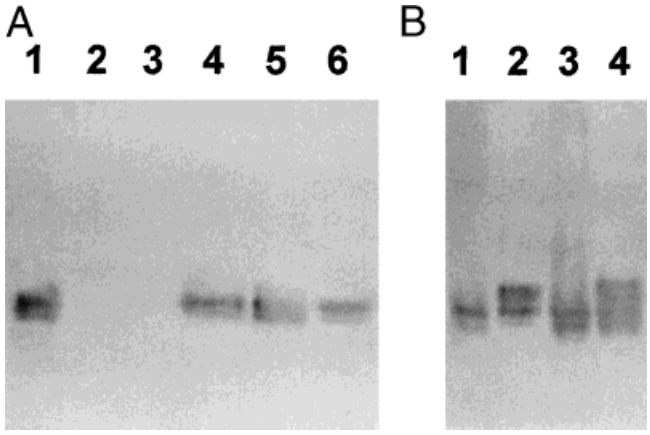

Fig. 2. Sequential extraction of DE from cauda epididymal spermatozoa. Sperm treated with 2 M NaCl were subsequently exposed to different extraction procedures. In all the cases the protein extracted from sperm were subjected to electrophoresis in nondenaturing nonreducing polyacrilamide gels (10%), transferred to nitrocellulose, and evaluated by Western blot. **A:** Purified protein DE detected as two bands corresponding to proteins D and E (lane 1); protein extracted from sperm by: PBS (lane 2), 50 mM glycine pH = 3 (lane 3), 100 mM Na_2CO_3 pH = 11 (lane 4), 6 M guanidine hydrochloride (lane 5), or 1% Triton X-100 (lane 6). Identical results were obtained with urea (data not shown). **B:** Protein extracted from sperm by 1% Triton X-100 (lane 1) or 100 mM DTT (lane 2). Purified DE (lane 3) and DTT-treated DE (lane 4).

extracted by high pH, guanidine hydrochloride, urea, and Triton X-100 (Fig. 2A). In all cases, the complete removal of DE occurred, judged by the lack of fluorescent labeling on treated spermatozoa (Fig. 1e–f).

The possibility that the tightly bound population could correspond to an intra-acrosomal, testicular protein was examined. However, Western blot experiments revealed no cross reaction of the anti-DE antibody with either testicular cytosol or protein extracts of testicular cells (data not shown).

Although the analysis of the complete amino acid sequence of DE indicates the absence of hydrophobic domains, it also predicts the potential formation of an amphipatic α-helix (amino acids 93–105), which could account for the insertion of the protein into the lipid bilayer. To explore this possibility, purified protein DE was incubated with different concentrations (0.1–1 mg) of PC, or PC-cholesterol unilamellar liposomes. After incubation, the samples were ultracentrifuged and the presence of DE in the pellet (liposomes) was evaluated. Results shown in Figure 3 indicated no difference in the emission spectra corresponding to liposomes incubated in the presence or absence of DE. The emission spectra corresponding to liposomes incubated with ionophore A23187, used as a control, evidenced the insertion of this compound (data not shown).

Considering the abundance of cysteine residues in DE, we explored the possible removal of the protein from the sperm surface by treatment of the cells with a disulfide-reducing agent. IIF revealed that DTT completely removed tightly bound DE from the sperm surface (data not shown). The protein extracted from sperm by this treatment presented a shift in its electrophoretic mobility compared to that extracted from the cells by Triton X-100 (Fig. 2B, lanes 1 and 2). This shift was also observed when purified DE was treated with the reducing agent (Fig. 2B, lanes 3 and 4).

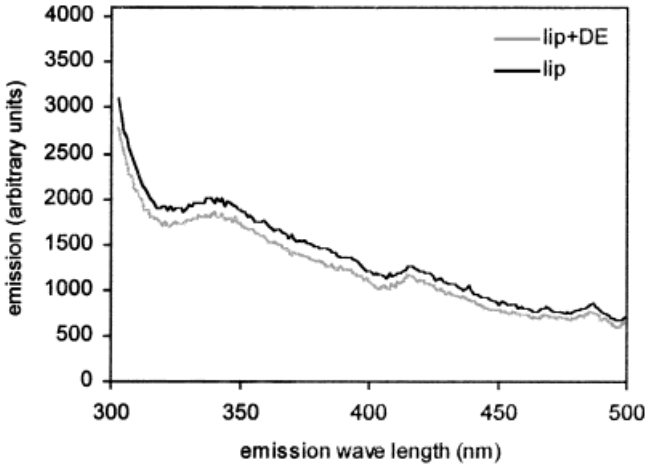

Fig. 3. Interaction of DE with liposomes. PC-liposomes were incubated with purified protein DE, ultracentrifuged, and the presence of DE in the pellet fraction (liposomes) was evaluated by measuring the emission fluorescence spectra of the sample (excitation: 280 nm, emission: 300–500 nm). The same results were obtained with PC-cholesterol liposomes.

Since DE associates with sperm while they are passing through the epididymis, we examined the presence of each of the two populations (i.e., released by, or resistant to, NaCl treatment) in the successive regions of the organ.

For this purpose, sperm recovered from the caput, corpus, and cauda epididymidis were exposed to 2 M NaCl, and the amount of DE present on the sperm prior and following the treatment were quantified by ELISA. Results indicated that while the population of DE resistant to the saline treatment (tightly bound) did not show significant differences among the three regions, the amount of DE released by NaCl significantly increased in the corpus and cauda regions of the organ (Fig. 4).

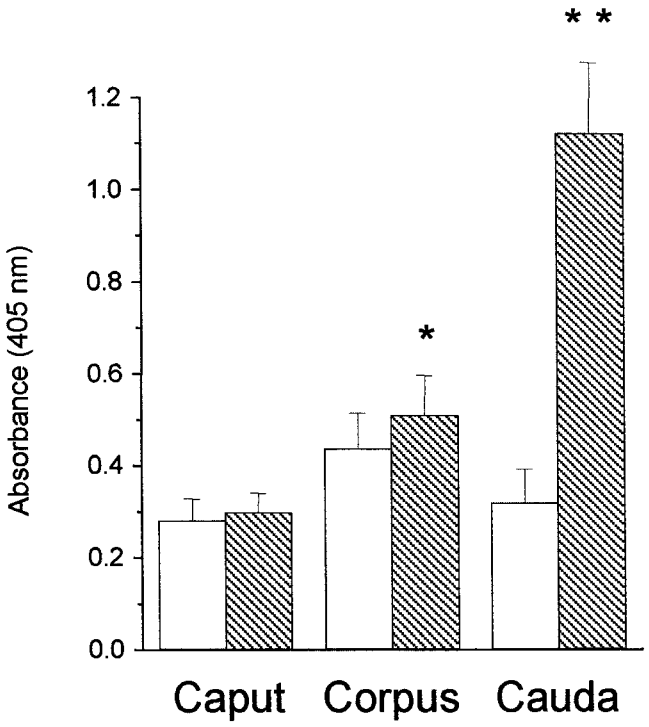

Fig. 4. Quantification of DE on spermatozoa during epididymal maturation. Sperm recovered from successive regions of the epididymis were exposed to 2 M NaCl treatment, and the amount of DE on sperm prior and following the treatment determined by ELISA using anti-DE as the first antibody. □, NaCl-resistant DE; ▨, NaCl-extractable DE (calculated as the difference between absorbance values before and after NaCl treatment). Results represent the mean value ± S.E. of five independent experiments. *$P < 0.05$ vs. caput; **$P < 0.05$ vs. corpus.

Behavior of the Two Populations of DE During Capacitation

As indicated above, a high proportion of DE is released from the sperm surface during capacitation (Kohane et al., 1980b), while a remnant of the protein persists even after the AR (Cameo et al., 1986). In order to examine whether the population released by the NaCl treatment (loosely bound) corresponded to the one released during capacitation, the amount of DE was determined by ELISA on cauda epididymal sperm either: (a) treated with NaCl (2 M); (b) capacitated for 5 hr; or (c) capacitated for 5 hr followed by exposure to NaCl (2 M). If the protein extracted from the sperm by ionic strength corresponded to that released during capacitation, a salt treatment subsequent to capacitation would not be able to remove additional DE from sperm. Figure 5 shows that while NaCl and capacitation released similar amounts of DE, no additional protein could be extracted by NaCl from previously capacitated sperm.

According to these results, the tightly bound population (resistant to 2 M NaCl) would correspond to the protein that remains on sperm after capacitation, and therefore to the one that migrates from the dorsal region to the equatorial segment of the acrosome with the occurrence of the AR (Rochwerger and Cuasnicú, 1992). To confirm this hypothesis, cauda epididymal sperm were first treated with NaCl (2 M) to remove the loosely bound protein, incubated under capacitating conditions for 5 hr, and finally subjected to IIF to evaluate the percentage of cells with DE on the equatorial segment. The high percentage of cells showing relocalization of DE after this treatment (Table 1) demonstrated the ability of the strongly bound population to migrate to the equatorial segment.

To investigate whether DE was modified after its migration from the dorsal to the equatorial segment, or as a consequence of capacitation, fresh sperm (dorsal localization) and ionophore-induced acrosome-reacted sperm (90% equatorial localization; Rochwerger and Cuasnicú, 1992), were treated with Triton X-100 to completely remove the protein from the sperm surface. Protein extracts and the capacitation medium containing the released protein DE were then subjected to electrophoresis and examined by Western blot. Results

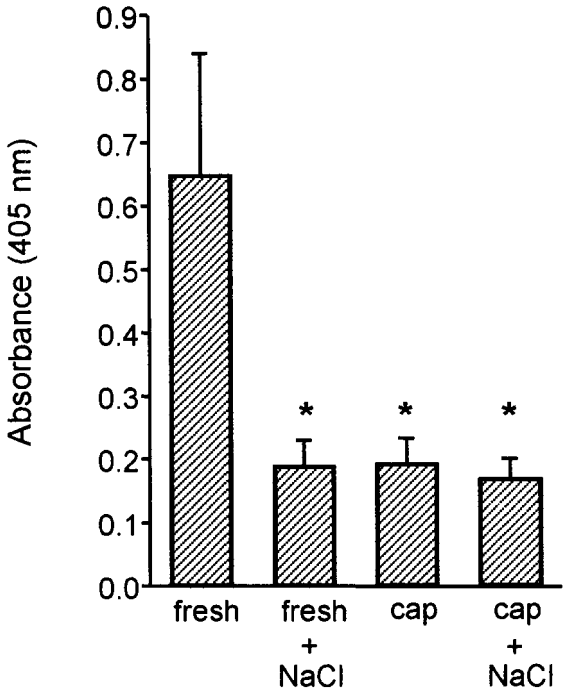

Fig. 5. Quantification of DE on sperm after their exposure to different treatments. Fresh (fresh) and capacitated (cap) sperm were incubated with or without 2 M NaCl, washed, and then plated on microtiter wells. The amount of DE present on sperm after these treatments was determined by ELISA using anti-DE as the first antibody. Results represent the mean value ± S.E. of four independent experiments. *$P < 0.001$ vs. fresh sperm.

TABLE 1. Localization of Tightly Bound DE After Incubation Under In Vitro Capacitating Conditions

Sperm treatment	Percent of sperm showing labeling on: Dorsal region	Percent of sperm showing labeling on: Equatorial segment
Capacitation, 0 hr	98 ± 2	1 ± 2
Capacitation, 5 hr	45 ± 4	53 ± 5
NaCl 2 M, capacitation, 0 hr	94 ± 3	6 ± 3
NaCl 2 M, capacitation, 5 hr[a]	7 ± 2	91 ± 2

[a]Cauda epididymal sperm were treated with 2 M NaCl and then incubated under capacitating conditions for 5 hr. Sperm were fixed and then subjected to IIF to determine the percentage of sperm with fluorescent labeling on the dorsal region or equatorial segment of the acrosome. Results represent the mean value ± S.E. of three independent experiments.

revealed no differences in the electrophoretic pattern among the three samples (Fig. 6).

DISCUSSION

As a first step towards understanding the interaction between DE and the sperm surface, the amino acid sequence of DE was determined by two different approaches: microsequencing of the purified protein and sequencing of a positive clone obtained by immunoscreening of a cDNA library with anti-DE. It is important to note that following the description of DE by our

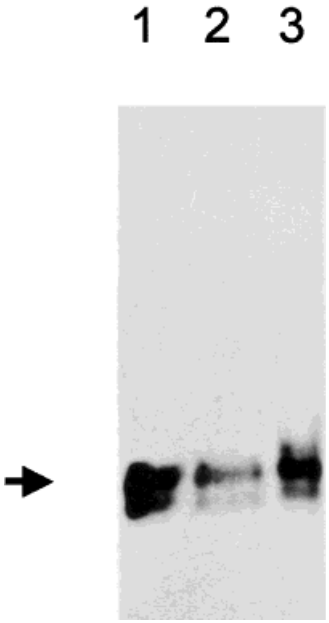

Fig. 6. Effect of capacitation and acrosome reaction on the electrophoretic mobility of protein DE. Proteins extracted with 1% Triton X-100 from either fresh (lane 1) or Ca^{2+} ionophore-acrosome-reacted sperm (lane 2), and proteins released to capacitation medium (lane 3) were separated by electrophoresis in 7.5% nondenaturing gels and analyzed by Western blot using anti-DE antibody.

laboratory in 1976 (Cameo and Blaquier, 1976), several laboratories reported the identification and/or characterization of rat epididymal proteins which received either the same (Brooks and Higgins, 1980) or different names, such as acidic epididymal protein (AEG; Lea et al., 1978), PES (Orgebin-Crist and Fournier-Delpech, 1982), or 32K protein (Wong et al., 1981). Although the similarities in many of their characteristics suggested that they might correspond to the same protein, the identity of these proteins was never confirmed. The results obtained by both approaches used in the present study indicated a 100% homology between protein DE identified in our laboratory and those previously cloned and sequenced by Brooks and colleagues (1986) and Charest and colleagues (1988), confirming that at least these three proteins correspond to the same molecule.

The analysis of the complete sequence of DE showed that it lacks hydrophobic domains and belongs to the CRISP (cysteine rich secretory proteins) family, containing 16 conserved cysteine residues. DE exhibits significant homology with mouse epididymal protein AEG1/CRISP1 (70%; Mizuki and Kasahara, 1992; Haendler et al., 1993), human epididymal protein ARP/CRISP1 (40%; Hayashi et al., 1996; Kratzschmar et al., 1996), mouse, guinea pig, human, and rat testicular protein TPX-1/CRISP2 (55–57%; Kasahara et al., 1989; Foster and Gerton, 1996; Kratzschmar et al., 1996; Maeda et al., 1998), as well as with a series of proteins not present in the male reproductive system, such as mouse and human salivary gland proteins CRISP-3/AEG 2 (62.5%, 55%; Mizuki and Kasahara, 1992;

Haendler et al., 1993; Kratzschmar et al., 1996), a protein from the Mexican lizard venom, helothermine (47%; Morrissette et al., 1995), a glycoprotein from human neutrophils, SGP28 (55%; Kjeldsen et al., 1996); and proteins from phylogenetically distant organisms like insects (Fang et al., 1988) and plants (Rigden and Coutts, 1988). However, besides the participation of epididymal protein DE in gamete fusion (Rochwerger et al., 1992) and the ability of helothermine to block ryanodine receptors in vitro (Morrissette et al., 1995), little is known about the functional role of other members of this family.

In an attempt to gain insights into the interaction of DE with the sperm surface, cauda epididymal sperm were subjected to different treatments known to remove peripheral and integral proteins. Results indicated that although treatment of mature sperm with 2 M NaCl was capable of removing a large amount of DE from sperm, part of the protein remained on the cell surface after this treatment, as judged by the presence of fluorescent labeling on the dorsal region of the sperm heads.

To examine the association of the strongly bound protein, sperm previously exposed to high ionic strength were subjected to different extraction procedures. Results indicated that while treatment of sperm with low pH was as ineffective as NaCl in removing the protein from the cells, DE could be completely extracted by agents that remove integral proteins. The possibility that this tightly bound population could correspond to an intra-acrosomal, testicular protein (i.e., testicular homologue TPX-1 or a testicular isoform of DE) was considered. The anti-DE antibody, however, does not cross-react with either testicular cytosol or protein extracts from testicular cells in Western blot, confirming previous reports of the lack of cross reaction of anti-DE with testicular cytosol (Garberi et al., 1979) and testicular tissue sections (Kohane et al., 1980a). Thus, although the possible presence of a testicular homologue/isoform of DE in the tightly bound population cannot be completely excluded, these results support the existence of a population of epididymal protein DE behaving as an integral protein.

Although the analysis of the amino acid sequence of DE revealed that the protein does not contain hydrophobic domains long enough to allow its insertion into the lipid bilayer (Brooks, 1987; Xu et al., 1997), the presence of a potential amphipatic α-helix led us to explore this possibility by examining the ability of DE to interact with liposomes. However, the lack of interaction between DE and the liposomes, at least under the conditions assayed, would not support a direct insertion of DE into the lipid bilayer.

The abundance of cysteine residues in the molecule raised the possibility that disulfide bonds might be involved in DE-sperm surface interaction. Although the finding that DTT was able to entirely remove the protein from sperm could first suggest the binding of DE to a membrane component by disulfide bonds, the complete extraction of the protein by other agents not capable of breaking covalent interactions (Triton X-100, guanidine hydrochloride, etc.) does not support this possibility. This conclusion is in agreement with results reported by Eberspaecher and colleagues (1995), indicating the absence of free-sulfhydryl groups in the recombinant mouse CRISP-1 protein, and suggesting that all cysteine residues are engaged in disulfide bonds. A possible explanation for the removal of the protein by DTT is the occurrence of a conformational change in the protein induced by the reduction of intramolecular disulfide bonds. Such conformational change is evidenced in the shift in electrophoretic mobility observed both in the purified protein treated with DTT and the protein extracted by this agent from mature sperm. Another possibility is that DTT reduces disulfide bonds in a sperm membrane component to which DE binds, causing the release of the epididymal protein from the sperm surface. In any case, our results suggest that the tightly bound population would correspond to a peripheral protein associated with the sperm surface by strong noncovalent interactions that involve disulfide bonds.

The nature of DE association with the sperm surface is a matter of controversy. Rankin and colleagues (1992), working with MEP 7, the mouse homologue of DE, concluded that the protein is predominantly loosely bound to the sperm surface. On the other hand, Moore and colleagues (1994), using the monoclonal antibody 4E9 which recognizes protein E (Xu and Hamilton, 1996), could not remove the protein by treatments that release peripheral proteins and suggested that the protein would be tightly associated with the sperm surface. The authors also demonstrated that the antigen is not bound to the membrane via a GPI linkage or to another protein that is linked in this way. Our results support the existence of both a major population of DE loosely associated with the cells by ionic interactions, and a minor population tightly bound to a sperm membrane component. This conclusion is in agreement with the results reported by Wong and Tsang (1982) for rat epididymal protein 32K, indicating that the protein presents two binding kinetics, i.e., one of high affinity but low capacity, and another one of lower affinity but higher capacity. The presence and biochemical nature of the sperm membrane component (i.e., protein or lipid) to which DE binds is currently being studied in our laboratory.

Since protein DE associates with the sperm surface during epididymal maturation, we examined at what point during this process sperm acquire each DE population. The finding that the amount of tightly-bound DE was not significantly different among the successive epididymal regions, while that corresponding to the loosely bound protein increased significantly in the corpus and cauda regions of the organ, suggests that DE would associate strongly with sperm in the proximal regions of the epididymis, and loosely during epididymal transit.

Several lines of evidence support the conclusion that the DE protein released during capacitation (Kohane et

al., 1980b) would correspond to the loosely bound population removable by ionic strength, while the one remaining after capacitation and migrating to the equatorial segment would correspond to the tightly bound population: 1. The amount of DE released from mature sperm during capacitation (65%) is not significantly different from that released by NaCl treatment (62%); 2. No further protein can be extracted by NaCl once sperm are capacitated, indicating that the loosely bound protein is released during capacitation, and 3. The majority of capacitated sperm lacking the loosely bound DE show the protein on the equatorial segment of the acrosome.

Interestingly, the proportion of sperm with DE on the equatorial segment after treatment with NaCl was considerably higher than the percentage usually obtained when sperm are capacitated without previous treatment (~50%). This is possibly due to the fact that NaCl treatment releases numerous proteins from the sperm surface in addition to DE, promoting the capacitation process. This would result in an increase in the proportion of cells undergoing the AR and, consequently, exhibiting DE on the equatorial segment.

Modification of a molecule during a physiological process has been previously reported (Phelps and Myles, 1987; Blobel et al., 1990; Jones et al., 1990). It was a likely possibility that DE also undergoes changes during either capacitation, AR, or its migration from the dorsal to the equatorial segment. However, no differences were found between the electrophoretic mobilities of the protein extracted from fresh sperm (DE localized on the dorsal region) or from ionophore-induced acrosome-reacted sperm (DE localized on the equatorial segment), or the protein released to the medium during capacitation. Although the occurrence of minor changes not detectable by the Western blot technique cannot be excluded, these results suggest that no major modifications occur in DE as a consequence of these biological processes.

In conclusion, the results obtained in the present work revealed the existence of two populations of DE: a major population, loosely bound to the sperm surface that is released during capacitation, and a minor population, tightly bound to the cells, that remains after capacitation, migrates to the equatorial segment with the AR, and therefore would correspond to the one with a role in the sperm-egg fusion process.

Two independent laboratories have recently reported the identification of a human epididymal secretory protein (ARP/hCRISP1) with 40% homology to DE (Hayashi et al., 1996; Kratzschmar et al., 1996). Several characteristics of this protein, such as its epididymal origin, secretory nature, molecular weight, and localization on the sperm head suggest its participation in the sperm-egg fusion process, as the rat counterpart (Hayashi et al., 1996). In view of the results of the present work, our laboratory is currently studying both the association of the human epididymal protein with the sperm surface as well as its participation in sperm-egg interaction.

ACKNOWLEDGMENTS

The authors thank Dr. David Hamilton for kindly providing the rat epididymal cDNA library, Dr. Alberto Darszon for his collaboration in the DE-liposome interaction assays supported by PLACIRH, and Dr. Lucrecia Calvo for stimulating discussions and constructive comments on the manuscript. This study was supported by a WHO grant (H9/181/R429) to P.S.C. D.J.C., D.A.E., and D.B. are recipients of Research Fellowships from the National Research Council of Argentina (CONICET). P.S.C. is a recipient of a Research Career Award from CONICET.

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