BIOLOGY OF REPRODUCTION **67,** 1225–1231 (2002)
DOI 10.1095/biolreprod.102.003657

Expression and Structure-Function Analysis of DE, a Sperm Cysteine-Rich Secretory Protein That Mediates Gamete Fusion[1]

Diego A. Ellerman, Vanina G. Da Ros, Débora J. Cohen, Dolores Busso, Mauro M. Morgenfeld, and Patricia S. Cuasnicú[2]

Instituto de Biología y Medicina Experimental, 1428 Buenos Aires, Argentina

ABSTRACT

Rat sperm epididymal glycoprotein DE belongs to the cysteine-rich secretory protein (CRISP) family and participates in sperm-egg fusion through its binding to complementary sites on the egg surface. To investigate the molecular mechanisms underlying the role of DE in gamete fusion, in the present work we expressed DE in a prokaryotic system, and examined the relevance of carbohydrates and disulfide bonds for the biological activity of the protein. Immunofluorescence and sperm-egg fusion assays carried out in the presence of recombinant DE (recDE) revealed that this protein exhibits the ability to bind to the DE-egg binding sites and to inhibit gamete fusion, as does native DE (nDE). Comparison of the proteins indicated, however, that the inhibitory ability of recDE was significantly lower than that of nDE. This difference would not be due to the lack of carbohydrates in the bacterially expressed protein because enzymatically deglycosylated nDE was as able as the untreated protein to inhibit gamete fusion. To examine whether disulfide bridges are involved in DE activity, the presence of sulfhydryls in nDE and recDE was evaluated by the biotin-maleimide technique. Results indicated that, unlike nDE, in which all cysteines are involved in disulfide bonds, recDE contains free thiol groups. Subsequent experiments showed that reduction of nDE with dithiothreitol significantly decreased the ability of the protein to inhibit gamete fusion. Together, these results indicate that whereas carbohydrates do not have a role in DE-mediated gamete fusion, disulfide bridges are required for full biological activity of the protein. To our knowledge, this is the first study reporting the relevance of structural components for the function of a CRISP member.

epididymis, fertilization, fusion, ovum, sperm

INTRODUCTION

Fertilization in mammals involves a sequence of specific cellular interactions between the sperm and the egg. After binding and penetration of the zona pellucida, spermatozoa bind to and fuse with the oolema. Evidence supports the idea that gamete fusion occurs through the interaction of complementary molecules localized on specific domains of the sperm and egg plasma membranes. Although little is known about the molecular events involved in gamete fusion, several proteins on spermatozoa and ova have been proposed to participate in this process [1, 2].

The epididymal sperm protein DE, a candidate molecule to mediate gamete fusion in the rat, was first described by our laboratory [3]. This protein of 32 kDa contains 10% carbohydrates [4, 5], is synthesized in an androgen-dependent manner by the proximal segments of the epididymis, and associates with the sperm surface during epididymal maturation [6, 7]. Originally localized on the dorsal region of the sperm head, DE migrates to the equatorial segment concomitantly with the occurrence of the acrosome reaction [8]. Sequential extraction of proteins from epididymal sperm revealed the existence of two populations of DE bound to sperm: a major population loosely associated with the cells, which is released from sperm during capacitation, and a tightly associated protein that remains after capacitation and migrates to the equatorial segment [9].

The relocation of DE to the equatorial segment, the region through which sperm fuses with the egg, together with experiments showing that the polyclonal anti-DE antibody significantly inhibited sperm penetration of zona-free rat eggs [10], suggested a role for this protein in sperm-egg fusion. Subsequent studies in which exposure of zona-free eggs to purified DE protein significantly reduced the percentage of penetrated eggs without affecting sperm-egg binding, supported the participation of this protein in gamete fusion through complementary sites on the egg surface [11]. Indirect immunofluorescence (IIF) studies showed that these binding sites are localized over the entire egg surface with the exception of the area corresponding to the plasma membrane overlying the meiotic spindle [11, 12], a region through which fusion rarely occurs [13, 14]. Thus, whereas DE localizes on the fusogenic region of the sperm head, the DE-binding components localize on the fusogenic area of the egg surface.

Cloning and sequencing of DE indicated that it belongs to a growing family of cysteine-rich secretory proteins (CRISPs) [9], and exhibits significant homology with two other epididymal proteins: mouse protein AEG-1/CRISP-1 [15, 16], and human protein ARP/hCRISP-1 [17, 18]. Recent results from our group provided evidence indicating participation of the mouse [19] and human [20] homologues in gamete fusion through complementary sites on the surface of corresponding eggs.

In addition to the epididymal proteins known as CRISP-1, members of the family include CRISP-2 (also called TPX-1), which is expressed in the testes of mice, humans, guinea pigs, and rats [21–23]; CRISP-3, which is detected in various cell types and tissues such as equine seminal vesicles [24, 25], salivary glands of mice and humans [18,

[1]This work was partially supported by grant H9/181/R429 from the World Health Organization to P.S.C. D.E., D.C., and D.B. are research fellowship recipients from the National Research Council of Argentina (CONICET). V.G.D. and M.M. are research fellowship recipients from the Latinoamerican Programme for Training and Research in Human Reproduction (PLACIRH). P.S.C. is the recipient of a Research Career Award from CONICET.

[2]Correspondence: Patricia S. Cuasnicú, Instituto de Biología y Medicina Experimental, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina. FAX: 54 11 4786 2564; e-mail: cuasnicu@dna.uba.ar

Received: 15 January 2002.
First decision: 12 February 2002.
Accepted: 26 April 2002.

ISSN: 0006-3363. http://www.biolreprod.org

26], murine pre-B cells [27], and human neutrophils [28]; and two nonmammalian venom proteins, helothermine [29, 30] and cysteine-rich venom protein (CRVP) [31]. As all CRISP members, DE contains 16 cysteine residues, 10 of which are clustered in the C-terminal region of the molecule, suggesting a fundamental role for these amino acids in the biological activity of the protein. However, so far, the structure-function relationship of DE remains unknown.

In an attempt to gain insights into the molecular mechanisms underlying the role of DE in sperm-egg fusion, and in particular to investigate the relationship between the structure and function of the protein, in the present work we have successfully expressed functional recombinant DE, and examined the relevance of carbohydrates and disulfide bonds for the biological activity of the protein.

MATERIALS AND METHODS

Construction of the DE-Expressing Plasmid

A fragment containing the coding sequence of DE was amplified by polymerase chain reaction (PCR) using a phage clone containing the full length cDNA as a template. Two oligonucleotide primers were designed on the basis of the published DE sequence [32] with *Bam*HI and *Pst*I restriction sites at the 5′ ends. The 28-base pair (bp) primers we used were forward, 5′-ACGGATCCCAAGATACCACTGATGAATG-3′; and reverse, 5′-CAACTGCAGACCTGATTATGTGGACTGG-3′. PCR amplification was performed using Deep-Vent polymerase (New England BioLabs, Beverly, MA) in a Perkin-Elmer thermocycler (Norwalk, CT). The 780-bp fragment obtained was then cloned into pMAL-p2 plasmid (New England BioLabs) using the *Bam*HI and *Pst*I sites to obtain the recombinant plasmid pMAL-DE.

Expression and Purification of Recombinant Proteins

BL21 bacteria were transformed with either pMAL, pMAL-DE, or pMAL-ARP, a plasmid containing the human homologue of protein DE [17] (kindly provided by Dr. Kasahara, Hokkaido University, Japan). BL21 cells were grown in Luria-Bertani medium (Life Technologies, Rockville, MD) containing 100 μg/ml ampicillin at 37°C to reach an OD_{600} between 0.5 and 0.6. Expression of proteins fused to the maltose binding protein (MBP) was subsequently induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG; ICN Biomedicals, Costa Mesa, CA) and incubation continued for 5 h at 25°C.

To analyze the periplasmic or cytoplasmic expression of the proteins, cells were pelleted by centrifugation at 4000 × *g* for 20 min and suspended in 30 mM Tris (pH 8.0), 1 mM EDTA, and 20% sucrose. After a new centrifugation, cells were incubated in ice-cold 5 mM $MgSO_4$ for 10 min, and centrifuged at 8000 × *g* for 20 min. Periplasmic proteins were present in the supernatant, whereas nonperiplasmic proteins were found in the pellet.

To purify the recombinant proteins, bacteria were grown in the presence of IPTG as described above, and the cells were pelleted by centrifugation and suspended in 20 mM Tris (pH 7.4), 1 mM EDTA, and 1 M NaCl, and lysed by mild sonication. The suspension was centrifuged at 9600 × *g* for 30 min, and the fusion protein was purified from the supernatant by affinity chromatography through an amylose-resin (New England BioLabs) column according to the manufacturer's instructions. Briefly, the sample was applied to the resin that had been equilibrated with the buffer described above, and washed with the same buffer. The bound protein was eluted with 10 mM maltose, and fractions containing the protein were pooled, dialyzed against rat fertilization medium (RFM) [33], and concentrated by filtration through Centriplus YM-10 (Millipore Corp., Bedford, MA).

Electrophoresis and Western Blot Analysis

Protein extracts from 80 μl of bacteria culture and purified recombinant DE protein (recDE; 3 μg) were separated by SDS-PAGE (10% acrylamide) along with broad-range protein markers (Life Technologies). Treated and untreated native DE protein (nDE; 0.2 μg) were subjected to eletrophoresis in 12% acrylamide gels according to the Laemmli method [34]. Proteins were transferred to nitrocellulose according to the method of Towbin et al. [35], and the membranes were probed with polyclonal anti-DE [36] or anti-MBP antibody (New England BioLabs) as previously described [9, 20].

Purification of Epididymal Protein DE

Native DE was purified following the biochemical procedures described elsewhere [4, 5]. They included 1) NH_4SO_4 precipitation, 2) ion-exchange chromatography in diethylaminoethyl cellulose, 3) gel filtration in Sephadex G-100 (Amersham Pharmacia Biotech, Buenos Aires, Argentina), and 4) affinity chromatography on Sepharose-Concanavalin A (Amersham Pharmacia Biotech). Using these procedures, a 95% pure protein with silver staining is obtained.

Deglycosylation of DE

DE protein (120 μg) was dissolved in 100 μl of 50 mM Na_2HPO_4 (pH 7.5) and incubated in the presence of 7500 units of Peptide N-glycosidase F (PNGase F, New England BioLabs) for 3 h at 37°C. After this period, 5000 units of fresh enzyme were added to the tube, and incubated for an additional 3 h at 37°C. DE protein was then dialyzed overnight against RFM. The protein-free control solution was subjected to the same treatments.

Detection of Sulfhydryls

Equimolar amounts of nDE, recDE, and MBP were treated with an excess of biotin-maleimide (Sigma Chemical Company, St. Louis, MO) for 1 h at 37°C in the presence of 2% SDS. While recDE and MBP were separated by electrophoresis in 7.5% acrylamide gels, nDE was subjected to electrophoresis in 10% acrylamide gels. Proteins were transferred to a nitrocellulose membrane, which was then stained in 0.1% Ponceau S (Sigma) in 5% (v/v) acetic acid for 10 min. After recording the results, the membrane was blocked with nonfat dried milk for 1 h at room temperature, incubated with avidin-peroxidase (1:500 in PBS, Sigma) for 1 h at room temperature, and after three washes with PBS, it was incubated with 0.4 mg/ml diaminobenzidine (Sigma) in 100 mM Tris (pH 7.5) and 0.01% v/v H_2O_2.

Reduction and Alkylation of DE

Native DE (200 μg) was incubated in 50 mM Tris (pH 8.5) containing 250 mM dithiotreitol (DTT; Sigma) for 2 h at 37°C. After this period, 0.1 volume of 1.5 M iodoacetic acid (IAA; Merck Química Argentina, Buenos Aires) was added, and the protein was incubated for 2 h at room temperature in the dark. Both DTT and IAA were eliminated by dialyzing the solution overnight against RFM. The protein-free control solution was subjected to the same treatments.

Recovery and Treatment of Gametes

Spermatozoa were obtained from the cauda epididymides of adult Sprague-Dawley rats, and were capacitated in RFM as previously described [8]. Metaphase II-arrested oocytes were collected from the oviductal ampullae of superovulated prepuberal female Sprague-Dawley rats. Cumulus cells were removed by incubating the egg-cumulus complex for 3 min in RFM containing 0.1% hyaluronidase (Type IV; Sigma), and zonae pellucidae were dissolved by treating the cumulus-free eggs with acid Tyrode solution (pH 2.5) for 10–20 sec [11].

For experiments involving the effect of proteins on sperm-egg fusion, zona-free eggs were incubated for 30 min in 100-μl drops (under oil) of RFM alone or containing either treated or untreated nDE, recDE, or control proteins (MBP and ARP). To examine the effect of proteins on sperm-egg binding, zona-free oocytes were incubated in 25 μM recDE or MBP for 30 min. For IIF experiments, zona-free rat eggs were incubated for 30 min in 6 μM recDE or MBP as a control.

Sperm-Egg Fusion Assays

Capacitated sperm were added to zona-free eggs ($0.5–2 \times 10^5$ cells/ml), and the gametes were coincubated for 3 h at 37°C in an atmosphere of 5% CO_2 in air. Eggs were then washed and analyzed for evidence of sperm penetration under phase-contrast microscopy (400×). Eggs were considered penetrated if a decondensing sperm head or two pronuclei and a sperm tail were present in the ooplasma. Alternatively, sperm-egg fusion was evaluated by incubating rat eggs with Hoechst 33342 (1 μg/ml) prior to insemination, and observing the transfer of the DNA-specific dye to the nucleus of the fused sperm as previously described [12]. Because both

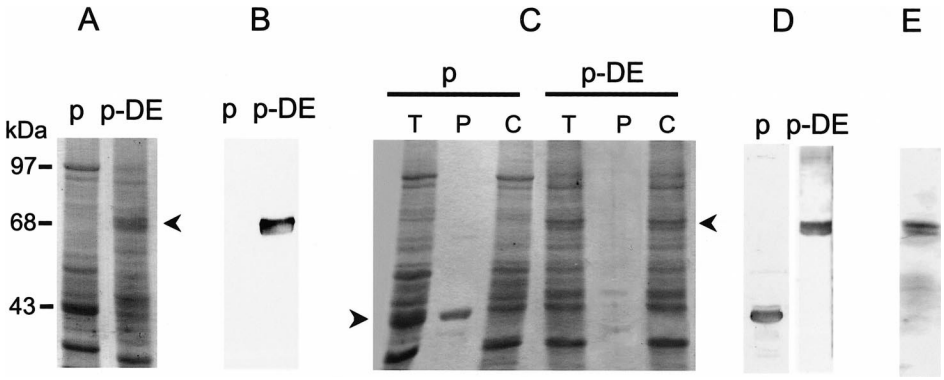

FIG. 1. Expression and purification of recDE. **A**) BL21 *E. coli* cells carrying the pMAL vector (p) or the pMAL-DE (p-DE) construction were grown in the presence of IPTG. Protein extracts from these bacterial cultures were separated by SDS-PAGE and stained with Coomassie brilliant blue (CBB). The presence of two close bands of ≈68 kDa in the lane corresponding to pMAL-DE is indicated by an arrowhead. **B**) Samples were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis using anti-DE as first antibody. **C**) Samples from bacteria transformed with pMAL or pMAL-DE corresponding to a total cell extract (T), or fractions containing the periplasmic (P) or cytoplasmic (C) proteins, were separated by SDS-PAGE and stained with CBB. Arrowheads indicate the position of MBP and recDE in the periplasmic and cytoplasmic fractions, respectively. **D**) The periplasmic fraction from bacteria transformed with pMAL and the cytoplasmic fraction from bacteria transformed with pMAL-DE were subjected to Western blot analysis using anti-MBP and anti-DE as first antibodies, respectively. **E**) Silver staining of recDE after purification by affinity chromatography.

methods gave identical results, the former procedure was preferable for a large number of samples.

For evaluation of sperm-egg binding, capacitated sperm ($0.5–2 \times 10^5$ cells/ml) were added to zona-free oocytes treated as described above. After an incubation period of 30 min, eggs were transferred serially through several drops of fresh RFM to remove loosely attached sperm. The eggs were then fixed in 2% glutaraldehyde in PBS for 30 min at room temperature, and the number of sperm bound per egg was determined.

Indirect Immunofluorescence

Treated zona-free rat eggs were fixed in 2% paraformaldehyde in PBS for 40 min at room temperature, and then washed several times with PBS containing 10 mg/ml BSA. Oocytes were then incubated in 5% normal goat serum in PBS for 30 min at 37°C, and exposed to either anti-DE (1:50) or anti-MBP (1:100) for 1 h at 37° C. After washing three times in PBS containing 10 mg/ml BSA and 0.02% Tween-20, the oocytes were incubated for 30 min at 37° C in fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Sigma). After three washings, oocytes were incubated for 2 min in Evans Blue, mounted in 90% glycerol in PBS, and examined with a Nikon Optiphot microscope (Nikon, Tokyo, Japan) equipped with epifluorescence optics.

Statistical Analysis

Effective concentration (EC_{50}) values were calculated by nonlineal regression using Prism 3.0 software (GraphPad Software, San Diego, CA). Differences between the effect produced by nDE and recDE were analyzed by the Wilcoxon rank test. The number of bound sperm per egg and the percentages of penetrated eggs were analyzed by the Student *t*-test. Results were considered significantly different at $P < 0.05$.

RESULTS

Expression of Recombinant DE Protein

In order to produce recombinant DE protein, the coding region for the mature protein (amino acids 20 to 246) [32] was amplified by PCR, subcloned into the pMAL expression vector, and introduced into BL21 *Escherichia coli* cells. Nonreducing SDS-PAGE analysis of *E. coli* culture lysates revealed the induction of two very close bands, with an apparent molecular weight of 68 kDa, matching the expected molecular weight for the fusion protein (DE, 26 kDa + MBP, 42 kDa) (Fig. 1A). These bands were absent in cells carrying the vector without the insert.

To confirm the presence of DE in the fusion protein, cell extracts were analyzed by Western blot using anti-DE as first antibody. Results indicated that the 68-kDa protein was specifically recognized by anti-DE because the antibody did not cross-react with any other protein present in extracts of bacteria containing pMAL without the insert (Fig. 1B).

The pMAL-p2 vector contains the leader peptide sequence that potentially directs the fusion protein to the bacterial periplasm. In order to determine the subcellular localization of both recDE and MBP, cytoplasmic and periplasmic fractions were analyzed by SDS-PAGE. Whereas MBP alone was successfully exported to the periplasm, recDE was associated to the cytoplasmic fraction (Fig. 1, C and D).

Although recDE was mainly associated with the insoluble fraction of the cell lysate when IPTG induction was conducted at 37°C, a substantial proportion of the protein could be recovered from the soluble fraction when expression was carried out at 25°C (data not shown). The soluble fraction was then passed through an amilose-resin column, and purification of the recombinant protein was monitored by SDS-PAGE and silver staining (Fig. 1E).

Biological Activity of recDE

Inhibition of sperm-egg fusion. Sperm-egg fusion is significantly inhibited by the presence of native DE protein during gamete coincubation. In order to examine whether the bacterially expressed protein exhibits this inhibitory ability, different concentrations (0.75–25 μM) of recDE were used in parallel to nDE in zona-free egg penetration assays. ARP, the human homologue of DE, also expressed as an MBP fusion protein, as well as MBP alone, were used as controls. Results showed that the presence of recDE in the incubation medium produced a dose-dependent reduction in the percentage of penetrated eggs with a significant effect observed at 1.2 μM, and maximum inhibition reached at 25 μM compared with 6 μM for nDE (Fig. 2). Both ARP and MBP, assayed at the highest concentrations (25 μM), did not affect egg penetration. Comparison between the recombinant and native protein indicated that the EC_{50} for recDE was significantly higher than that corresponding to nDE (8.3 μM vs. 3.1 μM; $P < 0.05$).

The decrease in the percentage of egg penetration was not due to a toxic effect of the fusion protein on sperm,

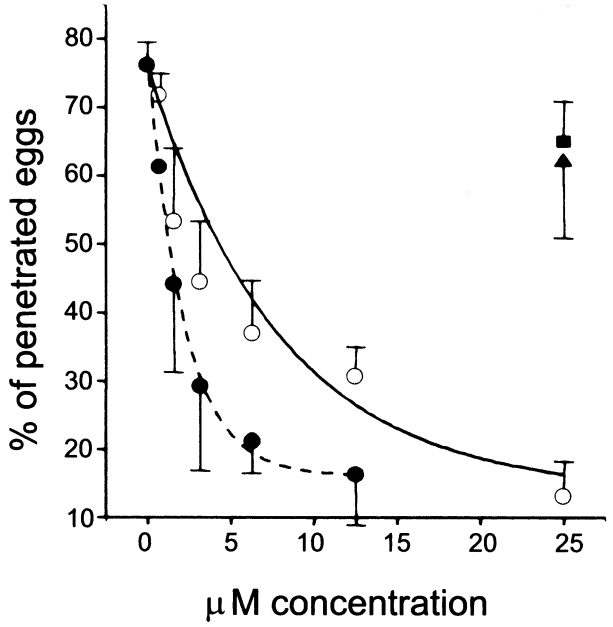

FIG. 2. Ability of recDE to inhibit sperm-egg fusion. Zona-free rat eggs were preincubated for 30 min with either nDE (closed circles), recDE (open circles), MBP (triangle), or recombinant human ARP (square) at the indicated concentrations, and then inseminated with capacitated sperm. The percentage of fertilized eggs was determined 3 h later. Each symbol represents the mean percentage of fertilized eggs from at least three independent experiments ± SEM.

because cells incubated with 25 μM recDE or MBP presented similar percentages of viability (56 ± 16 vs. 59 ± 18) and motility (52 ± 5 vs. 53 ± 6). In order to exclude the possibility that inhibition was due to an irreversible effect of the recombinant protein on egg penetrability, oocytes were incubated with 25 μM recDE or MBP for 2 h, washed in fresh medium to release the bound protein, and then inseminated with capacitated sperm. Oocytes exposed to recDE presented a high percentage of penetrability (82% ± 7%) that was not significantly different from control oocytes incubated with MBP (78% ± 15%).

Sperm-egg fusion comprises two steps: a first step of sperm-egg binding, and a second step of fusion itself. In order to evaluate whether recDE affected the first stage of the process, sperm-egg binding was evaluated after 30 min of gamete coincubation in the presence of 25 μM recDE or MBP. Results indicated that inhibition of gamete fusion was not due to an effect of recDE on sperm binding ability because neither the percentage of oocytes with bound sperm nor the mean number of sperm bound per egg were significantly different from controls (Table 1).

Binding of recDE to the egg surface. In order to confirm the binding of recDE to the DE complementary sites on the egg surface, zona-free eggs were incubated with the recombinant protein and then subjected to IIF using anti-DE or anti-MBP as first antibodies. Zona-free rat eggs exposed to MBP were used as controls. Results showed that oocytes incubated with recDE presented a patchy fluorescent labeling over the entire egg surface with the exception of an unlabeled area already described for those oocytes incubated with nDE [11] (Fig. 3, A and B). Oocytes incubated with recDE and then washed in fresh medium (Fig. 3C), as well as those incubated with MBP (Fig. 3D), were completely negative.

TABLE 1. Effect of recDE on sperm-oolema binding.

	Eggs with bound sperm*	Mean number of bound sperm/eggs*
recDE	68% ± 10% (63)	2.9 ± 0.4 (43)
MBP	65% ± 12% (67)	3.6 ± 0.8 (44)

* Number of analyzed oocytes in parentheses. Values correspond to the mean of three separate experiments ± SEM.

Importance of Carbohydrates for DE Activity

The results described above indicate that recDE was capable of inhibiting sperm-egg fusion, although with a lower efficiency than nDE. Because recDE was expressed in a prokaryotic system, its lower inhibitory ability could be due to the lack of glycosylation in the molecule. In order to evaluate the relevance of carbohydrates for the biological activity of DE, the native protein was treated with PNGase F, and then tested for its ability to inhibit sperm-egg fusion in vitro. A protein-free solution subjected to the same treatments was used as a control. Figure 4A shows the successful deglycosylation of DE, as evidenced by the higher electrophoretic mobility of the protein in nonreducing polyacrylamide gels. Results shown in Figure 4B indicate that the deglycosylated protein was as efficient as the nontreated protein in inhibiting sperm-egg fusion.

Importance of Disulfide Bridges for DE Activity

Recent evidence from our laboratory showing a shift in the electrophoretic mobility of DE after its exposure to DTT has suggested the involvement of intramolecular disulfide bonds in protein conformation [9]. Because recDE was expressed in the bacterial cytoplasm, an alternative explanation for its lower inhibitory activity could be a wrong

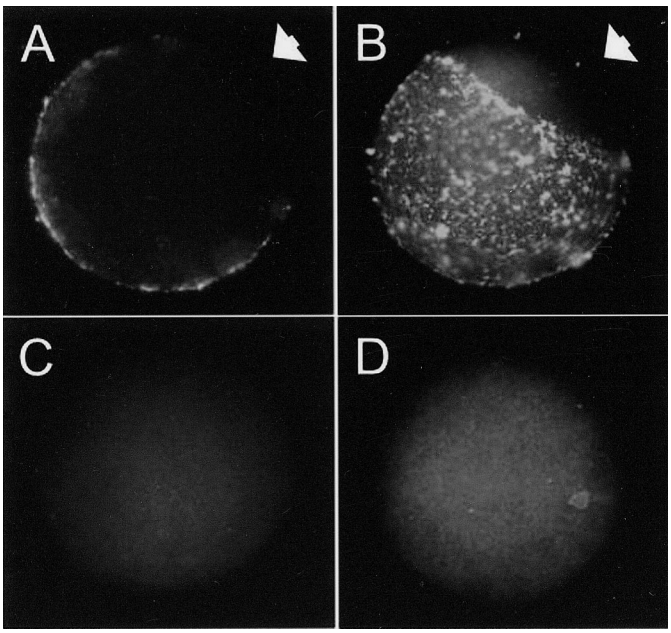

FIG. 3. Egg binding ability of recDE. **A** and **B**) Two different images of zona-free rat eggs exposed to 6 μM recDE, fixed, and then subjected to IIF using anti-MBP as first antibody. Note the presence of a fluorescence-free area (arrowheads). Identical results were obtained using anti-DE as first antibody. **C**) Zona-free eggs incubated with 6 μM recDE, washed, fixed, and then subjected to IIF using anti-MBP as first antibody. **D**) Zona-free eggs incubated with 6 μM MBP, fixed, and then exposed to anti-MBP. Magnification ×250.

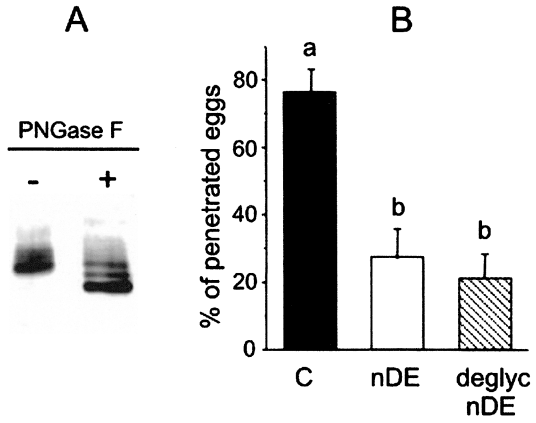

FIG. 4. Effect of deglycosylation on nDE activity. **A**) Native protein DE was deglycosylated by treatment with PNGase F in nondenaturing conditions. The untreated (−) and treated (+) proteins were separated by SDS-PAGE and revealed by Western blot analysis using anti-DE antibody. Purified nDE is detected as two bands corresponding to proteins D and E. The shift in the electrophoretic mobility confirmed the deglycosylation of DE. **B**) Zona-free rat eggs were preincubated in medium alone (C), or in medium containing 10 μg of nDE or deglycosylated nDE for 30 min and then inseminated with capacitated sperm. The percentage of penetrated eggs was determined after 3 h of coincubation. Each bar represents the mean value of at least three independent experiments ± SEM. a vs. b, $P < 0.05$.

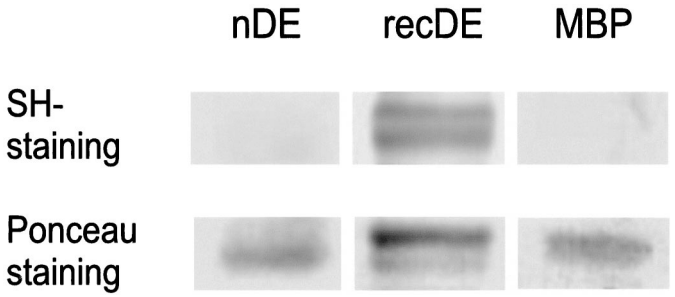

FIG. 5. Oxidative status of cysteines in nDE and recDE. Sulfhydryls in nDE, recDE, and MBP were labeled by treating the proteins with biotin-maleimide. Equimolar amounts of the proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with avidine-peroxidase and a peroxidase substrate (upper panel). Loading of each lane was verified by staining the membrane with Ponceau S (lower panel).

folding of the protein due to the lack of disulfide bridges. In order to evaluate this possibility, the presence of free sulfhydryl groups in both nDE and recDE was evaluated by labeling the molecules with biotin-maleimide. MBP, with no cysteines in its sequence, was used as a negative control. Equimolar amounts of the proteins were separated by SDS-PAGE, transferred to nitrocellulose, and visualized with avidin-peroxidase. As shown in Figure 5, no labeling was observed on nDE, which strongly suggests that all cysteines would be engaged in disulfide bonds. Conversely, recDE was found to exhibit a clear signal, which is indicative of the presence of free sulfhydryls.

Because a lower content of disulfide bonds in recDE might be responsible for the lower inhibitory ability of the protein, the relevance of disulfide bridges for DE activity was investigated. For this purpose, nDE was reduced with DTT, alkylated to avoid restoration of disulfides after DTT removal, dialyzed, and finally used in sperm-egg fusion assays. A protein-free solution subjected to the same treatments was used as a control. Results indicated that reduction of DE, confirmed by the shift in the electrophoretic mobility of the protein (Fig. 6A), significantly decreased the ability of DE to inhibit gamete fusion (Fig. 6B).

DISCUSSION

Evidence obtained so far supports the participation of rat epididymal DE protein and its mouse and human homologues in gamete fusion [12, 19, 20]. However, the molecular mechanism by which DE interacts with the complementary sites on the egg surface remains unknown. As part of a study to elucidate this mechanism, in the present work we successfully expressed DE protein in a prokaryotic system and examined the structure-function relationship of the protein.

Expression of DE was carried out using pMAL-p2, a vector designed for targeting the recombinant protein to the periplasmic space. RecDE, however, was detected as a double band in the bacterial cytoplasm. The failure of the protein to translocate to the periplasm might be explained by aspects of the protein structure, other than the presence of the leader peptide, being involved in the passage through the bacterial membrane [37, 38]. It has been reported that most of the recombinant proteins that fail to reach the periplasm either form inclusion bodies or are degraded [39, 40]. On the basis of this information, the presence of two close bands revealed by anti-DE immunoblotting might be due to mild proteolysis of recDE, because a substantial proportion of the recombinant protein was recovered from the soluble fraction.

In vitro experiments carried out in the presence of recDE revealed that the protein was able to inhibit zona-free egg penetration in a concentration-dependent manner. Inhibition was not due to a detrimental effect of the recombinant protein on any of the gametes, because sperm viability and motility were not different from controls, and normal levels of sperm penetration were observed in recDE-incubated

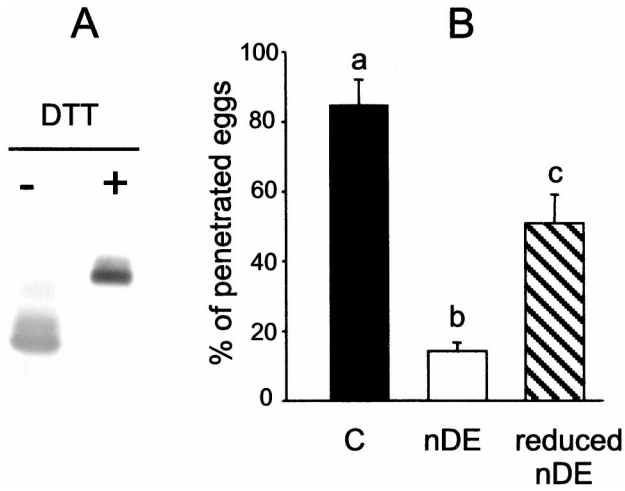

FIG. 6. Effect of reduction of disulfide bonds on nDE activity. **A**) Disulfide bridges in nDE were reduced by incubation with DTT, and sulfhydryls were then blocked by treatment with iodoacetic acid to avoid restoring S-S bonds. The unreduced (−) and reduced (+) proteins were separated by SDS-PAGE and revealed by Western blot using anti-DE antibody. The shift in the electrophoretic mobility confirmed protein reduction. **B**) Zona-free rat eggs were preincubated in medium alone (C) or in medium containing 20 μg of reduced or unreduced nDE for 30 min, and then inseminated with capacitated sperm. Three hours later, eggs were washed and examined for evidence of sperm penetration. Each bar represents the mean value of the percentage of penetrated eggs from at least three independent experiments ± SEM. Bars with different letters indicate statistical differences ($P < 0.05$).

eggs that had been subjected to washing prior to insemination. Neither the percentage of eggs with bound sperm nor the number of sperm bound per egg were affected by the presence of the recombinant protein, indicating that the inhibitory effect was subsequent to the sperm-oolema binding step. Finally, the finding that MBP as well as ARP (another member of the CRISP family also expressed as an MBP fusion protein) failed to inhibit egg penetration supports the specificity of the DE inhibitory effect.

Indirect immunofluorescence of recDE-incubated zona-free eggs showed the labeling pattern that had been previously observed in oocytes exposed to the native protein, i.e., fluorescent patches over the entire egg surface with the exception of an area corresponding to the plasma membrane overlying the meiotic spindle, a region through which fusion rarely or never occurs [13, 14]. The binding of recDE to the fusogenic region of the oolema was not mediated by the MBP portion of the fusion protein as judged by the fact that oocytes incubated with purified MBP were completely unlabeled. Together, these results indicate that recDE retains both its ability to recognize DE egg-complementary sites and to specifically inhibit gamete fusion.

Considering the lack of normal N-glycosylation in bacterially expressed proteins, the finding that the EC_{50} for recDE was significantly higher than that corresponding to nDE suggested that carbohydrates might contribute to the activity of the protein. In order to analyze this possibility, nDE was deglycosylated by digestion with PNGase F, an enzyme that removes the entire glycosydic core. The observation that deglycosylated DE was as efficient as the untreated protein in inhibiting gamete fusion, argues against any direct participation of the glycosydic residues in the interaction of DE with its egg-complementary sites. Nevertheless, the carbohydrate portion of the molecule might indirectly contribute to the function of the protein. One of the major roles of carbohydrates is to provide bulky, highly hydrophilic groups that help to maintain glycoproteins in solution during the folding process [41]. Misfolded proteins lacking N-oligosaccharides are generally found in the endoplasmic reticulum, where they are held by chaperones [42]. Evidence showed, however, that DE is normally secreted in the presence of tunicamycin [43], an inhibitor of N-glycosylation, suggesting that this protein can reach its native folding in the absence of carbohydrates. We cannot exclude the possibility that these residues might be important for other aspects of the protein such as its secretion by the principal cells of the epithelium, its resistance to proteases, its normal half life, or its binding to the sperm surface during epididymal maturation.

The high number and strict conservation of cysteines in CRISPs has suggested the existence of conserved intramolecular disulfide bonds, which would lead to a common tertiary structure with potentially important biological significance for this protein family [26, 44]. In view of the conformational change of DE observed after its reduction with DTT [9], an alternative explanation for the lower activity of recDE could be the absence of the proper disulfide bridges in the recombinant protein. In this regard, results clearly indicate that recDE contains free thiol groups, whereas in nDE, all or nearly all sulfhydryls would be involved in disulfide bonds. This result is not surprising considering that recDE was expressed in the bacterial cytoplasm, a reductor milieu [45].

In order to investigate the relevance of disulfide bonds for DE activity, the native protein was reduced, alkylated to avoid restoring of the disulfide bridges after DTT removal, and compared with the untreated protein for its ability to inhibit sperm-egg fusion. Considering that proper folding of sperm surface proteins is required for sperm-egg interaction [46, 47], the finding that DE reduction significantly affected its biological activity suggests the involvement of disulfide bonds in stabilizing the folding required for the interaction of DE with its egg-binding sites. The need for disulfide bonds in order for DE to exhibit full biological activity strongly supports the hypothesis that the lower content of disulfide bridges in recDE would be responsible for its lower ability to inhibit gamete fusion. The possibility that, in addition, incorrect disulfide bonds are present in recDE, cannot be excluded.

Although numerous proteins have been identified as members of the CRISP family, only two have so far been determined to have a well-characterized biological function. One is helothermine, a toxin obtained from the venom of the Mexican beaded lizard, which inhibits the ryanodine-type calcium channels [30]. The other is epididymal DE protein (and its mouse and human homologues), which participates in gamete fusion. Thus, DE protein constitutes a unique candidate of the CRISP family for performing structure-function analysis. To our knowledge, this is the first study reporting the relevance of structural components for the activity of a CRISP member.

Our data taken together indicate that whereas carbohydrates would not be involved in DE-mediated gamete fusion, disulfide bridges are required for the conformation and the biological activity of the protein.

Given the potential use of DE for fertility regulation [10, 48, 49], the availability of the recombinant protein will not only allow further research on the structure-function relationship of DE, but also provide an important tool to continue exploring the use of this protein for contraceptive development.

ACKNOWLEDGMENTS

We thank Dr. David Hamilton (University of Minnesota) for kindly providing the full-length DE λgt11 clone, and Dr. Alberto Kornblihtt (University of Buenos Aires) for his helpful advice on molecular biology procedures.

REFERENCES

1. Myles DG. Molecular mechanisms of sperm-egg membrane binding and fusion in mammals. Dev Biol 1993; 158:35–45.
2. Evans JP. Sperm disintegrins, egg integrins, and other cell adhesion molecules of mammalian gamete plasma membrane interactions. Front Biosci 1999; 4:D114–D131.
3. Cameo MS, Blaquier JA. Androgen-controlled specific proteins in rat epididymis. J Endocr 1976; 69:317–324.
4. Garberi JC, Kohane AC, Cameo MS, Blaquier JA. Isolation and characterization of specific rat epididymal proteins. Mol Cell Endocrinol 1979; 13:73–82.
5. Garberi JC, Fontana JD, Blaquier JA. Carbohydrate composition of specific rat epididymal protein. Int J Androl 1982; 5:619–626.
6. Kohane AC, Cameo MS, Piñeiro L, Garberi JC, Blaquier JA. Distribution and site of production of specific proteins in the rat epididymis. Biol Reprod 1980; 23:181–187.
7. Kohane AC, Gonzalez Echeverria F, Piñeiro L, Blaquier JA. Interaction of proteins of epididymal origin with spermatozoa. Biol Reprod 1980; 23:737–742.
8. Rochwerger L, Cuasnicu PS. Redistribution of a rat sperm epididymal glycoprotein after in vivo and in vitro capacitation. Mol Reprod Dev 1992; 31:34–41.
9. Cohen DJ, Rochwerger L, Ellerman DA, Morgenfeld M, Busso D, Cuasnicu PS. Relationship between the association of rat epididymal protein DE with spermatozoa and the behavior and function of the protein. Mol Reprod Dev 2000; 56:180–188.
10. Cuasnicú PS, Conesa D, Rochwerger L. Potential contraceptive use

of an epididymal protein that participates in fertilization. In: Alexander NJ, Griffin D, Spieler JM, Waites GMH (eds.), Gamete Interaction. Prospects for Immunocontraception. New York: Wiley-Liss; 1990: 143–153.
11. Rochwerger L, Cohen DJ, Cuasnicú PS. Mammalian sperm-egg fusion: the rat egg has complementary sites for a sperm protein that mediates gamete fusion. Dev Biol 1992; 153:83–90.
12. Cohen DJ, Munuce MJ, Cuasnicú PS. Mammalian sperm-egg fusion: the development of rat oolemma fusibility during oogenesis involves the appearance of binding sites for sperm protein ''DE''. Biol Reprod 1996; 55:200–206.
13. Johnson MH, Eager D, Muggleton-Harris A, Grave HM. Mosaicism in organisation of concanavalin A receptors on surface membranes of mouse eggs. Nature 1975; 257:321–322.
14. Ebensperger C, Barros C. Changes at the hamster oocyte surface from germinal vesicle stage to ovulation. Gamete Res 1984; 9:387–397.
15. Mizuki N, Kasahara M. Mouse submandibular glands express an androgen-regulated transcript encoding an acidic epididymal glycoprotein-like molecule. Mol Cell Endocrinol 1992; 89:25–32.
16. Eberspaecher U, Roosterman D, Krätzschmar J, Haendler B, Habenicht UF, Becker A, Quensel C, Petri T, Schleuning WD, Donner P, Kratzschmar J. Mouse androgen-dependent epididymal glycoprotein CRISP-1 (DE/AEG): isolation, biochemical characterization, and expression in recombinant form. Mol Reprod Dev 1995; 42:157–172.
17. Hayashi M, Fujimoto S, Takano H, Ushiki T, Abe K, Ishikura H, Yoshida M, Kirchhoff C, Ishibashi T, Kasahara M. Characterization of a human glycoprotein with potential role in sperm-egg fusion: cDNA cloning, immunohistochemical localization, and chromosomal assignment of the gene (AEGL1). Genomics 1996; 32:367–374.
18. Kratzschmar J, Haendler B, Eberspaecher U, Roosterman D, Donner P, Schleuning WD. The human cysteine-rich secretory protein (CRISP) family. Primary structure and tissue distribution of CRISP-1, CRISP-2 and CRISP-3. Eur J Biochem 1996; 236:827–836.
19. Cohen DJ, Ellerman DA, Cuasnicú PS. Mammalian sperm-egg fusion: evidence that epididymal protein DE plays a role in mouse gamete fusion. Biol Reprod 2000; 63:462–468.
20. Cohen DJ, Ellerman DA, Busso D, Morgenfeld M, Piazza A, Hayashi M, Young ET, Kasahara M, Cuasnicu PS. Evidence that human epididymal protein ARP plays a role in gamete fusion through complementary sites on the surface of the human egg. Biol Reprod 2001; 65:1000–1005.
21. Kasahara M, Gutknecht J, Brew K, Spurr N, Goodfellow PN. Cloning and mapping of a testis-specific gene with sequence similarity to a sperm-coating glycoprotein gene. Genomics 1989; 5:527–534.
22. Foster JA, Gerton GL. Autoantigen 1 of the guinea pig sperm acrosome is the homologue of mouse Tpx-1 and human TPX1 and is a member of the cysteine-rich secretory protein (CRISP) family. Mol Reprod Dev 1996; 44:221–229.
23. Maeda T, Sakashita M, Ohba Y, Nakanishi Y. Molecular cloning of the rat Tpx-1 responsible for the interaction between spermatogenic and Sertoli cells. Biochem Biophys Res Commun 1998; 248:140–146.
24. Magdaleno L, Gasset M, Varea J, Schambony AM, Urbanke C, Raida M, Topfer-Petersen E, Calvete JJ. Biochemical and conformational characterisation of HSP-3, a stallion seminal plasma protein of the cysteine-rich secretory protein (CRISP) family. FEBS Lett 1997; 420: 179–185.
25. Schambony A, Gentzel M, Wolfes H, Raida M, Neumann U, Topfer-Petersen E. Equine CRISP-3: primary structure and expression in the male genital tract. Biochim Biophys Acta 1998; 1387:206–216.
26. Haendler B, Kratzschmar J, Theuring F, Schleuning WD. Transcripts for cysteine-rich secretory protein-1 (CRISP-1; DE/AEG) and the novel related CRISP-3 are expressed under androgen control in the mouse salivary gland. Endocrinology 1993; 133:192–198.
27. Pfisterer P, Konig H, Hess J, Lipowsky G, Haendler B, Schleuning WD, Wirth T. CRISP-3, a protein with homology to plant defense proteins, is expressed in mouse B cells under the control of Oct2. Mol Cell Biol 1996; 16:6160–6168.
28. Kjeldsen L, Cowland JB, Johnsen AH, Borregaard N. SGP28, a novel matrix glycoprotein in specific granules of human neutrophils with similarity to a human testis-specific gene product and a rodent sperm-coating glycoprotein. FEBS Lett 1996; 380:246–250.
29. Mochca-Morales J, Martin BM, Possani LD. Isolation and characterization of helothermine, a novel toxin from *Heloderma horridum horridum* (Mexican beaded lizard) venom. Toxicon 1990; 28:299–309.
30. Morrissette J, Kratzschmar J, Haendler B, El-Hayek R, Mochca-Morales J, Martin BM, Jitandrakumar RP, Moss RL, Schleuning WD, Coronado R, Possani LD. Primary structure and properties of helothermine, a peptide that blocks ryanodine receptors. Biophys J 1995; 68:2280–2288.
31. Chang TY, Mao SH, Guo YW. Cloning and expression of a cysteine-rich venom protein from *Trimeresurus mucrosquamatus* (Taiwan habu). Toxicon 1997; 35:879–888.
32. Brooks DE, Means AR, Wright EJ, Singh SP, Tiver KK. Molecular cloning of the cDNA for androgen-dependent sperm-coating glycoproteins secreted by the rat epididymis. Eur J Biochem 1986; 161:13–18.
33. Kaplan R, Kraicer PF. Effect of elevated calcium concentration on fertilization of rat oocytes in vitro. Gamete Res 1978; 1:281–285.
34. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–685.
35. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979; 76:4350–4360.
36. Kohane AC, Piñeiro L, Blaquier JA. Androgen-controlled synthesis of specific protein in the rat epididymis. Endocrinology 1983; 112: 1590–1596.
37. Makrides SC. Strategies for achieving high-level expression of genes in *Escherichia coli*. Microbiol Rev 1996; 60:512–538.
38. Hannig G, Makrides SC. Strategies for optimizing heterologous protein expression in *Escherichia coli*. Trends Biotechnol 1998; 16:54–60.
39. Georgiou G, Valax P. Expression of correctly folded proteins in *Escherichia coli*. Curr Opin Biotechnol 1996; 7:190–197.
40. Baneyx F. Recombinant protein expression in *Escherichia coli*. Curr Opin Biotechnol 1999; 10:411–421.
41. Parodi AJ. Role of N-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation. Biochem J 2000; 348(part 1):1–13.
42. Marquardt T, Helenius A. Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. J Cell Biol 1992; 117: 505–513.
43. Brooks DE. Influence of incubation conditions, tunicamycin and castration on incorporation of [3H]mannose and [3H]fucose into rat epididymal glycoproteins in vitro. J Reprod Fertil 1983; 67:97–105.
44. O'Bryan MK, Loveland KL, Herszfeld D, McFarlane JR, Hearn MT, De Kretser DM. Identification of a rat testis-specific gene encoding a potential rat outer dense fibre protein. Mol Reprod Dev 1998; 50:313–322.
45. Bardwell JC. Building bridges: disulphide bond formation in the cell. Mol Microbiol 1994; 14:199–205.
46. Yanagimachi R, Huang TTF, Fleming AD, Kosower NS, Nicolson GL. Dithiothreitol, a disulfide-reducing agent, inhibits capacitation, acrosome reaction, and interaction with eggs by guinea pig spermatozoa. Gamete Res 1983; 7:145–154.
47. Ikawa M, Wada I, Kominami K, Watanabe D, Toshimori K, Nishimune Y, Okabe M. The putative chaperone calmegin is required for sperm fertility. Nature 1997; 387:607–611.
48. Perez Martinez S, Conesa D, Cuasnicú PS. Potential contraceptive use of epididymal proteins: evidence for the participation of specific antibodies against rat epididymal protein DE in male and female fertility inhibition. J Reprod Immunol 1995; 29:31–45.
49. Ellerman DA, Brantua VS, Martinez SP, Cohen DJ, Conesa D, Cuasnicu PS. Potential contraceptive use of epididymal proteins: immunization of male rats with epididymal protein DE inhibits sperm fusion ability. Biol Reprod 1998; 59:1029–1036.