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Sperm protein "DE" mediates gamete fusion through an evolutionarily conserved site of the CRISP family

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Abstract

The first member of the cysteine-rich secretory protein (CRISP) family was described by our laboratory in the rat epididymis, and it is known as DE or CRISP-1. Since then, numerous CRISPs exhibiting a high amino acid sequence similarity have been identified in animals, plants and fungi, although their functions remain largely unknown. CRISP-1 proteins are candidates to mediate gamete fusion in the rat, mouse and human through their binding to complementary sites on the egg surface. To elucidate the molecular mechanisms underlying CRISP-1 function, in the present work, deletion mutants of protein DE were generated and examined for their ability to bind to the rat egg and interfere with gamete fusion. Results revealed that the egg-binding ability of DE resides within a 45-amino acid N-terminal region containing the two motifs of the CRISP family named Signature 1 and Signature 2. Subsequent assays using synthetic peptides and other CRISPs support that the egg-binding site of DE falls in the 12-amino-acid region corresponding to Signature 2. The interesting finding that the binding site of DE resides in an evolutionarily conserved region of the molecule provides novel information on the molecular mechanisms underlying CRISP-1 function in gamete fusion with important implications on the structure–function relationship of other members of the widely distributed CRISP family.
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Introduction

The cysteine-rich secretory protein (CRISP) family is a large group of secreted proteins with molecular weights of about 20–30 kDa, characterized by the presence of sixteen conserved cysteine residues, ten of which are clustered in the C-terminal portion of the molecule. The first member of this evolutionarily highly conserved family was originally described by our laboratory in the rat epididymis (Cameo and Blaquier, 1976), and it is known as protein DE or CRISP-1 (Brooks et al., 1986; Cohen et al., 2000b). Since then, other members of the CRISP family have been identified in different mammalian tissues: CRISP-2, also known as Tpx-1, which is expressed in the testis and seems to be synthesized exclusively in the developing spermatids (Hardy et al., 1988; Kasahara et al., 1989; O'Bryan et al., 1998), CRISP-3, with a wider tissue distribution than the other CRISPs including saliva, prostate, pancreas, pre-B cells, human neutrophils, thymus, colon and ovary (Haendler et al., 1993; Kjeldsen et al., 1996; Udby et al., 2005), and the recently described CRISP-4 which is exclusively expressed in the epididymidis (Jalkanen et al., 2005). There are also non-mammalian CRISP proteins in venoms from lizards, snakes and snails (Morrissette et al., 1995; Milne et al., 2003; Yamazaki and Morita, 2004) and in eggs and embryos from *Xenopus* (Olson et al., 2001; Schambony et al., 2003). More distantly related proteins such as plant pathogenesis related 1 (PR-1) proteins, insect allergens (V5) and human Golgi-associated plant pathogenesis related protein (GAPR-1) exhibit significant homology to the N-terminal domain of CRISPs but lack the C-terminal cysteine-rich region (Fernandez et al., 1997; Henriksen

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et al., 2001; Serrano et al., 2004). Altogether, these proteins are grouped into the CAP (*C*RISP/*A*ntigen5/*PR*-1) superfamily.

Although the number of identified members of this family has steadily increased during the last years, only few of them have so far been determined to have a well-characterized biological function (Rochwerger et al., 1992; Morrissette et al., 1995; Milne et al., 2003). The first functional role for a CRISP member was described for epididymal glycoprotein DE (Rochwerger et al., 1992). After its secretion into the epididymal lumen, DE associates with the sperm surface during epididymal maturation (Kohane et al., 1980a,b) and migrates from the dorsal to the equatorial segment of the acrosome concomitantly with the occurrence of the acrosome reaction (Rochwerger and Cuasnicu, 1992). The relocation of DE to the equatorial segment, the fusogenic region of the sperm head, opened the possibility of a role for this protein in gamete fusion. Results showing that exposure of zona-free eggs to purified DE produced a significant reduction in the percentage of egg penetration without affecting the first step of sperm–egg-binding indicated that this protein participates in an event subsequent to binding and leading to fusion, through complementary sites on the egg surface. The DE-binding sites were localized to the fusogenic area of the egg surface (Rochwerger et al., 1992), and their temporal expression on the oocyte correlated with the acquisition of egg fusion ability during oogenesis (Cohen et al., 1996). DE exhibits high homology with two other epididymal proteins known as mouse AEG-1/CRISP-1 (Mizuki and Kasahara, 1992; Eberspaecher et al., 1995) and human ARP/hCRISP-1 (Hayashi et al., 1996; Kratzschmar et al., 1996), both of which are also involved in gamete fusion through binding sites on the surface of the corresponding eggs (Cohen et al., 2000a, 2001).

The mechanisms by which these epididymal proteins mediate sperm–egg fusion are still unknown. Structure–function studies performed by our laboratory using both native and bacterially expressed recombinant DE showed that the activity of this protein does not involve carbohydrates and resides in the polypeptidic region of the molecule (Ellerman et al., 2002). While disulfide bonds seem to be required for full biological activity of DE, the analysis of the amino acid sequence of the protein indicated the lack of functional domains that could explain its involvement in gamete fusion.

In order to get a better understanding of the molecular mechanisms underlying the functional role of DE, the present work was aimed towards the identification of the egg-binding site within the molecule. For this purpose, deletion mutants of DE as well as synthetic peptides were examined for their ability to bind to the egg surface and interfere with gamete fusion. Interestingly, our results show that the egg-binding site of DE resides in the region corresponding to an evolutionarily conserved motif of the CRISP family.

Materials and methods

Animals

Adult (age, 90–120 days) male and immature (age, 26–29 days) female Sprague–Dawley rats were maintained at 23°C with a 12L:12D cycle. Experiments were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* approved by the National Institute of Health (NIH).

Production of recombinant DE fragments and synthetic peptides

DE fragments were amplified by PCR using a vector containing the DE full-length cDNA as template (Ellerman et al., 2002). Several oligonucleotide primers were designed on the basis of the published sequence of DE (Brooks et al., 1986; Charest et al., 1988), and restriction sites for different enzymes and/or a stop codon at the 5′ ends were included:

Primer 1: 5′ ACGGATCCCAAGATACCACTGATGAATG 3′ (*Bam*HI)
Primer 2: 5′ CAACTGCAGACCTGATTATGTGGACTGG 3′ (*Pst*I)
Primer 3: 5′ TCTACAAGCTTAATTGCCACCAGGACAA 3′ (*Hin*dIII, *stop*)
Primer 4: 5′ AAGGATCCGGTGGCAATTATGTAGGAAG 3′ (*Bam*HI)
Primer 5: 5′ TAGGATCCAGGTGCATTTACAATGACAG 3′ (*Bam*HI)
Primer 6: 5′ TCGACAAGCTTCATTTCTGAGCGTTCACATA 3′ (*Hin*dIII, *stop*)
Primer 7: 5′ GCGGATCCAAAGTTGGTGTTAAAGTCGGA 3′ (*Bam*HI)
Primer 8: 5′ ACTCTGCAGTTATGGGCCGAAACCGAAGAC 3′ (*Pst*I)

The PCR amplifications were performed using Deep-Vent polymerase (New England BioLabs, Beverly, MA) in a Perkin-Elmer thermocycler (Norwalk, CT) (5 cycles at 40°C, plus 25 cycles at 50°C), and the following combinations of primers were used for each construct: primers 1 and 3 for Fragment 1 (F1); primers 4 and 2 for Fragment 2 (F2); primers 5 and 2 for Fragment 3 (F3); primers 5 and 3 for Fragment 4 (F4); primers 1 and 6 for Fragment 5 (F5); primers 5 and 8 for Fragment 6 (F6); and primers 7 and 3 for Fragment 7 (F7). Cloning into the pMAL-c2 vector (New England Biolabs) as well as protein production and subsequent purification were performed as previously described (Ellerman et al., 2002). To check the in-frame insertion of the fragments, each clone was sequenced using malE and M13/pUC sequencing primers (New England BioLabs) which anneal outside the insertion site (DNA Sequencing Facility, BRC, Cornell University, Ithaca, NY). Expression of the different fragments as maltose-binding protein (MBP)-fused proteins was analyzed by Western blot using anti-DE or anti-MBP as primary antibodies as previously described (Ellerman et al., 2002). The molecular weight of the detected bands corresponded to those expected for each fragment: F1: 60.7 kDa; F2: 50.4 kDa; F3: 61.2 kDa, F4: 53.5 kDa; F5: 49.3 kDa; F6: 48.5 kDa; F7: 47.5 kDa. MBP, used as control in different experiments, was expressed following the same procedure.

Biotin-conjugated synthetic peptides were prepared by Yale University Peptide Synthesis Facility (New Haven, CT). Peptide 1 (P1): GHYTQVVWNST; Peptide 2 (P2): FYVCHYCPGGNY; and scrambled Peptide 2 (scP2): VGCNYGPCHYYF.

Recovery and treatment of gametes

Spermatozoa

Sperm recovered from cauda epididymides were placed in a conical tube, covered with 2 ml of RFM (Kaplan and Kraicer, 1978) and allowed to swim up at 37°C (Rochwerger and Cuasnicu, 1992). For capacitation, aliquots of the upper sperm layer were added to 400 μl of media previously placed in tissue culture wells (16 mm; Costar, Cambridge, MA), at a final concentration of 0.5–1×10^6 sperm/ml. Sperm suspensions were then incubated under paraffin oil at 37°C for 5 h in an atmosphere of 5% CO_2 in air.

For evaluation of motility, 10 μl of sperm suspension was placed on pre-warmed slides, and the percentage of motility determined subjectively under a light microscope.

Eggs

Prepuberal female rats were superovulated by injection (s.c.) of eCG (equine chorionic gonadotropin, 20 UI; Syntex, Argentina) followed by the administration (s.c.) of hCG (human chorionic gonadotropin, 25 UI; Sigma) 48 h later. Metaphase II oocytes were collected from the oviducts of superovulated females 15 h after hCG injection. Cumulus cells were removed

by incubating the egg–cumulus complex for 3 min in RFM containing 0.1% hyaluronidase (Sigma, Type IV), and zonae pellucidae were dissolved by treating the cumulus-free eggs with acid Tyrode's solution (pH 2.5) for 10–20 s (Rochwerger et al., 1992).

For indirect immunofluorescence (IIF) experiments, zona-free eggs were incubated for 30 min in RFM containing 6 μM of either recombinant DE (Ellerman et al., 2002), recombinant fragments, MBP, mouse Tpx-1 (Busso et al., 2005), human ARP (Cohen et al., 2001) or helothermine (provided by Dr. Possani) or 6 to 30 μM of the synthetic peptides. For experiments involving the effect of different molecules on sperm–egg fusion, zona-free eggs were incubated in medium alone or containing different concentrations of either the recombinant proteins or fragments, or peptides (dissolved in dimethyl sulfoxide (DMSO)), for 30 min prior to insemination.

Indirect immunofluorescence

Eggs incubated with either proteins, fragments or peptides were fixed in 2% paraformaldehyde in PBS for 45 min at room temperature and then washed several times with PBS containing 4 mg/ml BSA (PBS–BSA 4). For detection of recombinant DE or DE fragments, fixed eggs were incubated in 5% normal goat serum (NGS) in PBS–BSA 4 for 30 min at 37°C and then exposed to either anti-DE (Kohane et al., 1983) (1:50 in 1% NGS) or anti-MBP (1:100). The same steps were followed for the rest of the proteins tested using the corresponding antibodies: anti-TPX1 (1:100, (Busso et al., 2005)), anti-ARP (1:100, (Cohen et al., 2001)), anti-helothermine (1:100, provided by Dr. Possani). All incubations were carried out at 37°C for 1 h. After washing 3 times in PBS–BSA 4, eggs were incubated for 30 min at 37°C in FITC-conjugated goat anti-rabbit antibody (1:50 in PBS–BSA 4; Sigma). For detection of synthetic peptides, fixed eggs were washed in PBS–BSA 4 and incubated with goat anti-biotin antibody (1:100 in PBS–BSA 4; Vector Laboratories Inc., Burlingame, CA) for 1 h at 37°C. After washing, eggs were incubated with FITC-conjugated rabbit anti-goat antibody (1:100 in PBS–BSA 4; Sigma). In all cases, eggs were washed, mounted in 90% glycerol in PBS and examined with a Nikon Optiphot microscope (Nikon, Tokyo, Japan) equipped with epifluorescence optics (250×).

Evaluation of sperm–egg fusion

Sperm–egg fusion was evaluated by one of the following assays:

(a) Sperm–egg penetration assay: capacitated sperm were added to treated zona-free eggs at a final concentration of $0.5–2 \times 10^5$ cells/ml, and gametes were co-incubated for 3 h at 37°C in an atmosphere of 5% CO_2 in air. Eggs were then washed in fresh medium to remove loosely adherent sperm, mounted on slides and analyzed for evidence of sperm penetration under a phase-contrast microscope (400×). Eggs were considered penetrated if a decondensing sperm head or two pronuclei and a sperm tail were present in the ooplasm.

(b) Hoechst dye transfer technique: this technique was performed as previously described (Cohen et al., 1996). Briefly, zona-free eggs were incubated in 500 μl of fresh RFM containing 1 μg/ml Hoechst 33342 (Sigma). After 5 min incubation at 37°C, eggs were washed four times for 10 min in 500 μl of fresh medium and then exposed to the fragments. After 30 min, eggs were inseminated with capacitated spermatozoa ($0.5–2.0 \times 10^5$ cells/ml) and gametes were co-incubated for 30 min. Eggs were then fixed with 2% paraformaldehyde in PBS, washed, mounted on slides and examined under a UV microscope for evidence of sperm–egg fusion. An egg was considered to have fused with sperm when at least one brightly stained sperm nucleus could be observed on its surface. The validity of the dye transfer technique was tested by inseminating zona-free eggs in Ca^{2+}-free medium, a condition under which fusion does not occur (Yanagimachi, 1978). For evaluation of the effect of F7 on egg penetrability, Hoechst-loaded eggs were exposed to the fragment for 30 min, washed, transferred to a drop of fresh medium and then inseminated as described above.

Statistical analysis

The percentages of egg penetration and fusion as well as sperm motility were analyzed by chi-square test. Results were considered to be significantly different at $P < 0.05$.

Results

The DE-egg-binding ability is within the N-terminal domain of the molecule

Considering that the amino acid sequence of rat DE lacks known functional domains that could explain its role in gamete

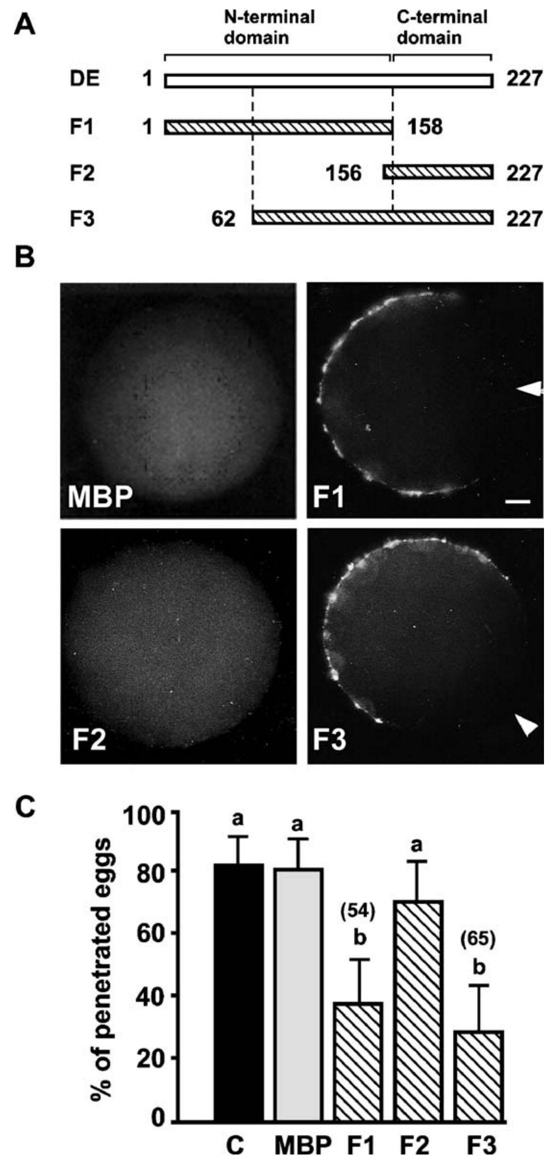

Fig. 1. Biological activity of fragments F1, F2 and F3. (A) Schematic diagram of the entire rat DE protein and the generated recombinant fragments. The numbers indicate the first and last residue for each fragment. (B) Zona-free eggs were incubated with 6 μM MBP, F1, F2 or F3, fixed and subjected to IIF using anti-MBP as primary antibody. Note the presence of a fluorescent labeling with a negative area (arrowheads) in those eggs incubated with F1 and F3. Similar results were obtained when anti-DE was used as primary antibody. Scale bar = 10 μm. (C) Zona-free eggs were pre-incubated in medium alone (C) or in medium containing 60 μM MBP, F1, F2 or F3 for 30 min and then inseminated with capacitated sperm. The percentage of penetrated eggs was determined after 3 h of co-incubation. Each bar represents the mean value of at least three independent experiments ± SEM. a vs. b, $P < 0.05$. The number in parentheses above each bar indicates the percentage of inhibition of the corresponding treatment compared to control (C).

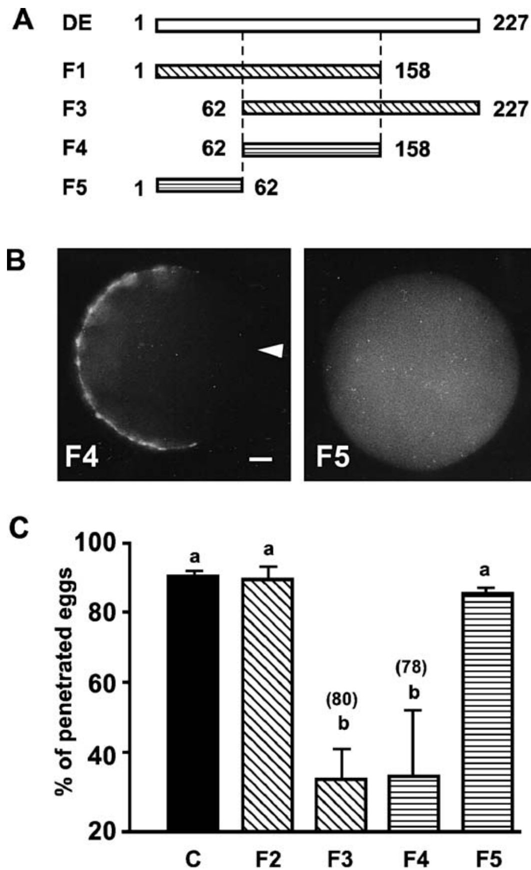

Fig. 2. Biological activity of fragments F4 and F5. (A) Schematic diagram of the generated fragments. The numbers indicate the first and last residue corresponding to each fragment. (B) Zona-free eggs were incubated with 6 μM F4 or F5, fixed and subjected to IIF using anti-MBP as primary antibody. The arrowhead shows the negative area. Scale bar = 10 μm. (C) Zona-free eggs were pre-incubated in medium alone (C) or in medium containing 60 μM F2, F3, F4 or F5 for 30 min and then inseminated with capacitated sperm. The percentage of penetrated eggs was determined after 3 h of co-incubation. Each bar represents the mean value of at least three independent experiments ± SEM. a vs. b, $P < 0.05$. The number in parentheses above each bar indicates the percentage of inhibition of the corresponding treatment compared to control (C).

fusion, we generated a first series of recombinant fragments based on the structural domains previously described for the recombinant mouse DE homologue (Eberspaecher et al., 1995). The structural analysis of this murine protein reveals the presence of a loose N-terminal domain, covering 75% of the protein and containing three disulfide bonds, and a compact C-terminal domain with the remaining ten conserved cysteines. In view of this, the following recombinant fragments were generated: Fragment 1 (F1), spanning from amino acid 1 to 158, corresponding to the N-terminal domain of DE, Fragment 2 (F2) (156–227), corresponding to the C-terminal domain, and Fragment 3 (F3) (62–227), containing the 16 cysteines of the molecule (Fig. 1A).

In order to examine the egg-binding ability of the generated fragments, rat zona-free eggs were incubated with equimolar concentrations (6 μM) of each fragment or MBP as a control and then fixed and subjected to IIF using anti-DE or anti-MBP as primary antibodies. Results shown in Fig. 1B indicated that, while eggs incubated with MBP did not exhibit fluorescent staining, those incubated with F1 or F3 showed the same fluorescent pattern previously described for the entire recombinant DE protein, i.e. a clear labeling localized over the egg surface with the exception of an area that remained unstained. By contrast, eggs incubated with F2 were completely unlabeled as the controls.

To establish whether binding of F1 and F3 to the eggs was capable of inhibiting gamete fusion, zona-free eggs were pre-incubated with each fragment, inseminated with rat capacitated sperm and then examined for evidence of sperm incorporation into the ooplasm, an unequivocal evidence of the occurrence of

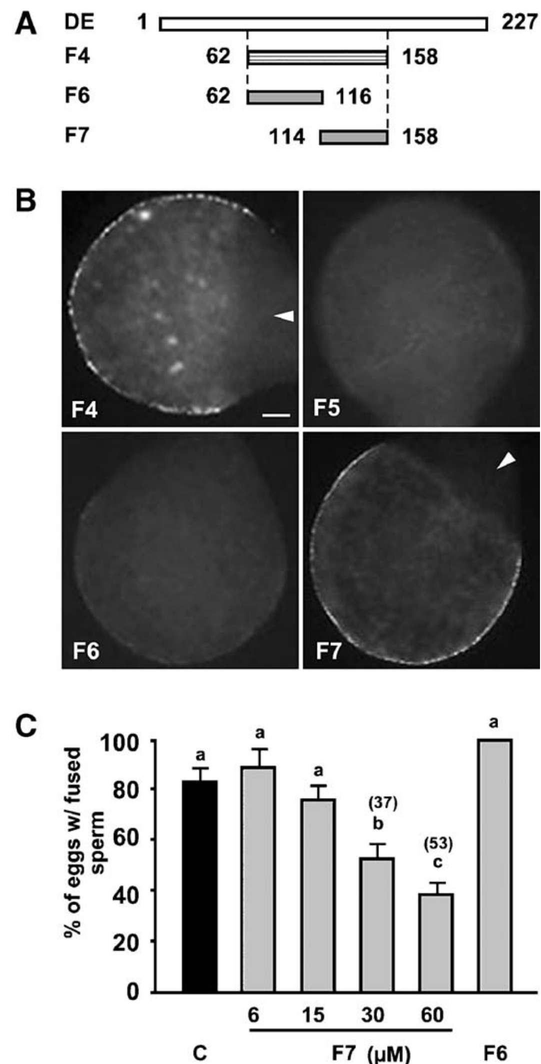

Fig. 3. Biological activity of fragments F6 and F7. (A) Schematic diagram of the generated fragments. The numbers indicate the first and last residue contained in each construct. (B) Zona-free eggs were incubated with 6 μM F4, F5, F6 or F7, fixed and subjected to IIF using anti-MBP as primary antibody. The arrowheads show the negative area in the eggs. Scale bar = 10 μm. (C) Zona-free eggs pre-loaded with Hoechst 33342 (1 μg/ml) were incubated for 30 min in medium alone (C) or in medium containing either different concentrations of F7 (6 to 60 μM) or 60 μM F6 and then inseminated with capacitated sperm. After 30 min of gamete co-incubation, eggs were fixed, mounted on slides and examined under UV microscope for evidence of sperm–egg fusion. Each bar represents the mean value of three independent experiments ± SEM. a vs. b, $P < 0.05$; a vs. c, $P < 0.001$. The numbers in parentheses above each bar indicate the percentage of inhibition compared to control (C).

gamete fusion. When the three fragments were tested at the concentration previously used for IIF (6 μM), none of them either individually or combined with each other was able to inhibit egg penetration (data not shown). However, when used at 60 μM, both F1 and F3 produced a significant reduction in the percentage of penetrated eggs compared to the control, not observed in the presence of MBP or F2 (Fig. 1C). These results indicated that F1 as well as F3 were able to both bind to the egg surface and inhibit sperm–egg fusion.

The DE-egg-binding ability resides in a 45-amino-acid region

The finding that the two fragments capable of binding to the egg, F1 and F3, shared an amino acid region led us to generate Fragment 4 (F4), corresponding to this region in common (62 to 158). As a control, we generated Fragment 5 (F5) corresponding to the portion of F1 not present in F3 (1 to 62) (Fig. 2A).

Evaluation of the egg-binding ability of these two fragments by IIF indicated that, while eggs exposed to 6 μM F5 did not show fluorescent labeling, those incubated with the same concentration of F4 showed a strong fluorescent labeling similar to that observed with F1 and F3 (Fig. 2B). These results indicated that the egg-binding ability of DE was contained within the 97-amino-acid region corresponding to F4. To confirm that binding of F4 to the egg was also capable of inhibiting gamete fusion, a sperm–egg fusion assay was performed in the presence of F4 or F5 using F2 and F3 as negative and positive controls, respectively. As shown in Fig. 2C, while the presence of 60 μM F5 or F2 did not produce

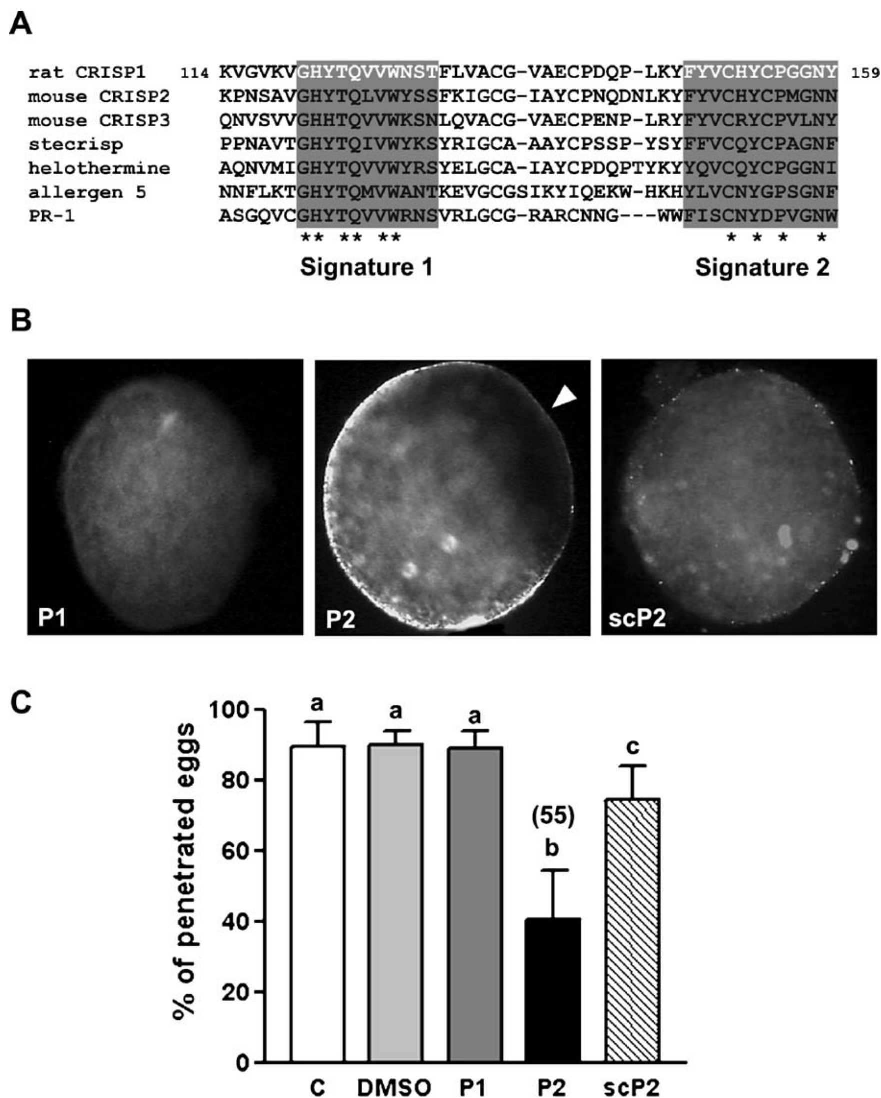

Fig. 4. Design and egg-binding ability of P1 and P2. (A) Signature 1 and Signature 2 containing regions of CAP representative members are boxed in gray. The amino acid sequences corresponding to Peptides 1 and 2 are shown in white. Asterisks indicate complete identity of all proteins. (B) Zona-free eggs were incubated with 30 μM of biotinylated P1, P2 or scP2, fixed and subjected to IIF using anti-biotin as primary antibody. Note the presence of a fluorescent labeling with a negative area (arrowhead) in eggs incubated with P2. Scale bar = 10 μm. (C) Zona-free eggs were pre-incubated in either medium alone (C), medium containing 1% DMSO or medium containing 60 μM P1, P2 or scP2 for 30 min and then inseminated with capacitated sperm. The percentage of penetrated eggs was determined after 3 h of co-incubation. Each bar represents the mean value of four experiments ± SEM. a vs. b, $P < 0.0001$, b vs. c, $P < 0.001$, a vs. c, $P > 0.05$ (not significantly different). The number in parentheses above the bar indicates the percentage of inhibition of P2 compared to control (C).

any effect on egg penetration compared to the control in medium alone, the presence of an equimolar concentration of F4 produced a significant reduction in the percentage of penetrated eggs, similar to that observed for eggs incubated with F3.

To examine in more detail the egg-binding ability of F4, two new recombinant fragments, F6 (62 to 116) and F7 (114 to 158), were generated (Fig. 3A) and examined for their ability to bind to the egg surface by IIF using F4 and F5 as controls. Results showed that, while oocytes incubated with F6 were completely negative, those incubated with F7 presented a fluorescent labeling similar to that observed with F4 (Fig. 3B).

Having established the ability of F7 to recognize and bind to the egg surface, sperm–egg fusion assays were performed in the presence of this fragment to test its ability to interfere with gamete fusion. In this case, however, a detrimental effect of F7 (60 μM) on sperm motility was observed at the end of the 3 h co-incubation period. In view of this, lower concentrations of F7 were tested, and sperm–egg fusion was examined by the Hoechst dye transfer technique which specifically evaluates the occurrence of gamete fusion after only a 30 min co-incubation period. Under these conditions, F7 did not affect sperm motility at any of the concentrations tested (i.e. F7 (60 μM): 55% ± 5 vs. C: 63% ± 6, NS) and produced a significant and concentration-dependent reduction in the percentage of eggs with fused sperm not observed by exposure to F6 (Fig. 3C). When the fusion inhibitory ability of F2 and F4 was evaluated by this technique, results consistent with those previously observed with the egg penetration assay were obtained (data not shown).

Considering the detrimental effect of F7 on sperm motility, experiments were performed to exclude the possibility that the inhibitory effect of F7 on gamete fusion was due to a toxic effect of the fragment on the eggs. For this purpose, zona-free eggs were incubated for 30 min in medium containing 60 μM F7 or in medium alone as a control, washed and then inseminated with capacitated sperm. Evaluation of gamete fusion by the Hoechst dye transfer technique indicated no difference between groups in the percentage of eggs with fused sperm (F7 and then washed: 80 ± 9, C: 96 ± 6; $n = 3$, NS).

The DE-egg-binding ability is located in Signature 2 of the CRISP family

The results obtained supported the idea that the biological activity of DE would reside in the 45-residue region spanning from amino acid 114 to 158. The analysis of this region revealed that it contains two motifs, denominated Signature 1 and Signature 2 and recognized by the PROSITE database as feature motifs of the CAP superfamily (Fig. 4A). To investigate whether these motifs were involved in the egg-binding ability of F7, two synthetic biotinylated peptides with the amino acid sequences corresponding to Signature 1 and Signature 2 in DE were produced: Peptide 1 (P1): GHYTQVVWNST and Peptide 2 (P2): FYVCHYCPGGNY (Fig. 4A). The ability of P1 and P2 to bind to the egg surface was tested by incubation of zona-free eggs with different concentrations of the peptides (6, 15 and 30 μM), fixation and exposure to an anti-biotin antibody and a corresponding FITC-conjugated secondary antibody. Results showed a complete negative labeling of eggs incubated with P1 at all concentrations tested (Fig. 4B). On the other hand, eggs incubated with 15 and 30 μM P2 presented the strong fluorescent labeling previously observed for all the active fragments. To examine whether the sequence of P2 was critical for its activity, a scrambled P2 peptide was synthesized (scP2: VGCNYGP-CHYYF) and used in IIF assays. In this case, with the exception of few fluorescent spots, no labeling on the egg surface was observed at any concentration (Fig. 4B). To examine whether the binding of P2 to the egg was capable of interfering with gamete interaction, sperm–egg fusion assays were performed in the presence of P1, P2 and scP2. When the peptides were tested at 15 or 30 μM, none of them was able to inhibit egg penetration (data not shown). When used at 60 μM, P1 and scP2 did not produce any effect on egg penetration compared to controls (medium alone or medium with DMSO), while P2 produced a significant reduction in the percentage of penetrated eggs (Fig. 4C).

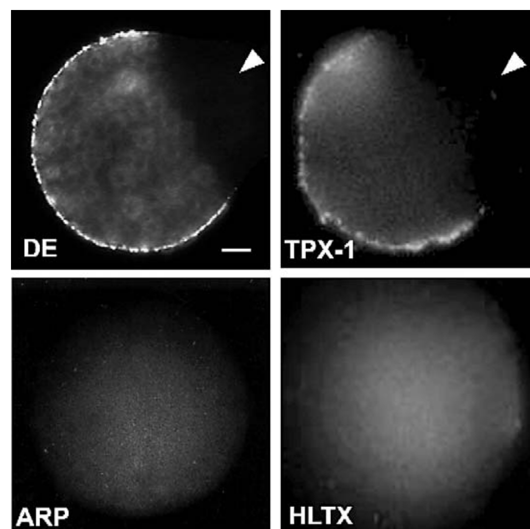

Fig. 5. Egg-binding ability of CRISP proteins. Zona-free rat eggs were incubated with 6 μM of rat recombinant DE (DE), mouse recombinant Tpx-1 (TPX-1), human recombinant ARP (ARP) or helothermine (HLTX) for 30 min, fixed and subjected to IIF using anti-DE, anti-TPX-1, anti-ARP or anti-helothermine as primary antibodies, respectively. Note the presence of a fluorescent labeling with a negative area (arrowheads) in eggs incubated with DE or Tpx-1. Scale bar = 10 μm.

These results strongly suggested that the ability of DE to bind to the egg surface resides in the 12-amino-acid region corresponding to Signature 2 of the CRISP family. To study the specificity of this interaction, we analyzed the ability of several CRISP proteins to interact with rat eggs as well as the amino acid sequence of their corresponding Signature 2 regions. As shown in Fig. 5, while mouse Tpx-1 was capable of binding to the rat egg, human ARP and helothermine were unable to recognize the rodent gamete. In correlation with this, the Signature 2 region presented only two substitutions in mouse Tpx-1 and four in both human ARP and helothermine, when compared to Signature 2 in rat DE (Table 1). The same analysis was applied to previous data indicating that rat DE is able to

Table 1
Analysis of the egg-binding ability of CRISP proteins in correlation with the amino acid sequence of their Signature 2 regions

CRISP protein	Egg	Egg-binding ability [a]	Signature 2 sequence in CRISP proteins (12 aa) [b]	No. of different aa compared to rat DE
Rat DE/CRISP-1	Rat	+	FYVCHYCPGGNY	–
Mouse Tpx-1/CRISP-2	Rat	+	FYVCHYCPMGNN	2/12
Human ARP	Rat	−	LYVCHYCHEGND	4/12
Helothermine	Rat	−	YQVCQYCPGGNI	4/12
Rat DE/CRISP-1	Mouse	+	YYVCHYCPVGNY [c]	2/12
Rat DE/CRISP-1	Human	−	LYVCHYCHEGND [d]	4/12

[a] Zona-free eggs were exposed to 6 μM of each tested protein, fixed and then subjected to IIF using the corresponding antibodies (anti-DE, anti-TPX1, anti-ARP, anti-helothermine or anti-MBP).
[b] The amino acids different to those in rat DE Signature 2 are underlined.
[c] The sequence shown corresponds to Signature 2 in mouse AEG protein.
[d] The sequence shown corresponds to Signature 2 in human ARP protein.

bind to the mouse but not to the human egg (Cohen et al., 2000a, 2001). In this case, the comparison revealed that the Signature 2 sequence in rat DE presents only two substitutions (one of them by a conservative amino acid) when compared to that of its mouse homologue (protein AEG) and four substitutions when compared to human ARP (Table 1).

Discussion

CRISPs are found across a broad variety of living forms and exhibit diverse biological functions. However, the molecular mechanisms underlying these functions remain unknown for most of the CRISP family members. One of these proteins is epididymal glycoprotein DE, a candidate molecule to mediate sperm–egg fusion through its binding to complementary sites on the egg surface (Rochwerger et al., 1992). To elucidate the molecular mechanisms involved in this interaction, in the present work, a series of recombinant fragments of DE were expressed in a prokaryotic system and evaluated for their ability to both bind to the egg surface and interfere with gamete fusion. IIF and sperm–egg fusion experiments using the first series of fragments (F1–F3) revealed that the egg-binding ability of DE would be contained within the N-terminal domain of the molecule. Subsequent experiments using a new series of recombinant fragments (F4–F7) circumscribed this activity to a region of 45 amino acids (114–158) within this domain.

Eggs incubated with those fragments capable of binding to their surface (F1, F3, F4, F7) exhibited the fluorescent patterns previously observed for both native and entire recombinant DE (Rochwerger et al., 1992; Ellerman et al., 2002). In all cases, the fragments capable of binding to the egg surface were also able to compete with sperm and inhibit gamete fusion while those unable to bind to the egg (F2, F5, F6) also failed to interfere with gamete interaction. The consistent correlation between these two parameters supports the results obtained and validates the use of these biological assays for evaluation of DE activity. The observation that a higher concentration of fragments was required to inhibit gamete fusion compared to that used for the entire recombinant protein suggests that other regions of the molecule, although not essential, might be contributing to the proper configuration of the egg-binding site.

The analysis of the 45-amino-acid region exhibiting the egg-binding domain revealed that this fragment contained the two hallmark motifs of the CAP superfamily: Signature 1 and Signature 2. The use of synthetic peptides corresponding to each of these motifs (P1 and P2, respectively) in IIF and sperm–egg fusion assays indicated that P2 but not P1 was capable of binding to the egg and interfering with gamete fusion. The lack of both egg labeling and fusion inhibition observed with a peptide containing the same amino acids than P2 but in a different order (scrambled P2) confirmed the relevance of this region for the binding of DE to the egg.

Together, the results obtained using both the recombinant fragments and the synthetic peptides indicated that the activity of DE resides in the 12-amino-acid region corresponding to Signature 2. To our knowledge, this is the first work describing a functional role for the motif of the CRISP family and succeeding in delimiting the activity of a CRISP protein to such a small region. This result becomes particularly interesting considering that DE has been shown to be a good candidate for contraceptive technique development. Evidence from our laboratory indicated that immunization of male and female rats with purified DE (Cuasnicú et al., 1990; Ellerman et al., 1998) or the recombinant protein (unpublished results) leads to an efficient contraceptive effect in both sexes. Thus, the finding that the activity of DE resides in only 12 amino acids represents an important contribution for the future design of new and safer fertility regulating methods.

The recently reported crystal structure of a CRISP family member, stecrisp, revealed that this protein contains two domains: a plant pathogenesis-related domain (PR-1) and a cysteine-rich domain (CRD), connected by a short hinge (Guo et al., 2005). The significant similarity in CRISP family members ensures that other CRISPs will possess very similar three-dimensional structures as stecrisp, the reason why the extended N-terminal and the compact C-terminal domains of these proteins are now also referred to as PR-1 and CRD, respectively. According to this, our results indicated that the egg-binding ability of DE resides within the PR-1 domain. Similar results were reported for Tpx-1/CRISP-2, a testicular homologue of DE proposed to mediate binding between spermatogenic and Sertoli cells through its 101 N-terminal amino acid residues (Maeda et al., 1998). Nevertheless, the involvement

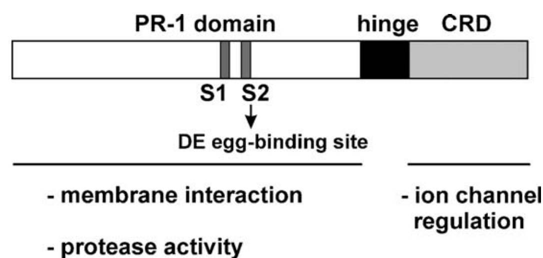

Fig. 6. Schematic diagram illustrating the structural domains of CRISP proteins and their relationship with proposed functional activities. CRISP members contain a plant pathogenesis-related (PR-1) domain and a cysteine-rich domain (CRD), connected by a short hinge. The two evolutionarily conserved regions of the CRISP family, named Signature 1 (S1) and Signature 2 (S2), are located within the PR-1 domain. Based on the results of the present work, the egg-binding site of DE resides in S2. The proposed functions for CRISP members include an ion channel regulatory activity in the CRD, and both a protease activity and a membrane-interacting activity, in the PR-1 domain.

of the CRD domain in other potential functions of DE cannot be excluded. Recent evidence indicates that CRISP proteins from snake venoms (Yamazaki and Morita, 2004) as well as mouse Tpx-1/CRISP-2 (Gibbs et al., 2006) possess an ion channel regulatory activity located in a region of the CRD. In this regard, it is interesting to mention that DE has been shown to have an inhibitory activity on sperm protein tyrosine phosphorylation (Roberts et al., 2003), a capacitation-associated event which depends on the regulation of several ion channels (Visconti et al., 2002). In view of the existence of a population of DE loosely associated to sperm which is released from the cell during capacitation (Cohen et al., 2000b), it is possible that DE also acts as a decapacitation factor, regulating ion channels through its cysteine-rich domain.

The crystallographic analysis of several PR-1 containing proteins including tomato P14a (Fernandez et al., 1997), vespid Ves v 5 (Henriksen et al., 2001), recombinant GAPR-1 (Serrano et al., 2004) and snake venom stecrisp (Guo et al., 2005) revealed a unique α–β–α sandwich fold of PR-1 domain and a cleft with two highly conserved histidines and glutamates suggested as a putative active site. Moreover, recent evidence indicating that Tex31, a snail PR-1 protein, has a proteolytic activity (Milne et al., 2003) implied the possibility of PR-1 domain as an enzymatic module. The failure to identify enzymatic activities in other CRISPs (Guo et al., 2005), however, opened the possibility of additional functions for this domain. In this regard, our findings indicating that the egg membrane binding ability of DE is within the PR-1 domain and localized in a region different from the proposed putative active site strongly support the idea that a great variance of functions and active sites may be present in the PR-1 domain. Thus, although sharing a common structural organization, it is likely that different members of this family have evolved to perform a variety of functions that rely on different regions of the molecule, i.e. the ion channel regulatory activity in the CRD, and both the protease activity and the membrane-interacting activity (this work) in the PR-1 domain (Fig. 6). The relevance of the highly conserved three-dimensional structure of CRISP members for these different functions remains to be established.

Results from our laboratory indicated that, as rat DE, the mouse and human epididymal homologues also play a role in gamete fusion (Cohen et al., 2000a, 2001). Moreover, we have recently described that human TPX1 would participate in this event of the fertilization process (Busso et al., 2005). Since all these proteins are closely related members of the CRISP family with a common biological activity, it is likely that they operate through a similar molecular mechanism. However, the finding that the egg-binding ability of DE corresponds to a highly conserved sequence in these proteins raised the question about how this common region might possess the necessary specificity for interacting with the different eggs. To address this issue, the ability of several CRISPs to bind to the egg was analyzed in correlation with the amino acid sequence of their corresponding Signature 2 regions. This analysis revealed that, in those cases where a protein was able to bind to the egg of another species (i.e. mouse Tpx-1 to rat eggs or rat DE to mouse eggs), the sperm proteins from the two species exhibited a higher sequence homology in their Signature 2 region than that observed in those cases where the proteins were unable to interact with the eggs (i.e. human ARP to rat egg, helothermine to rat egg, DE to human egg). These observations support the participation of Signature 2 in gamete fusion and suggest that differences in the amino acid sequence of this region may be responsible for the specificity of the binding of each CRISP to its target egg.

In addition to the interaction of these CRISP proteins with the egg, other members of the CAP superfamily have been proposed to associate with the membrane of different cells i.e. *Na*-ASP-2 with phagocytes (Ehlers, 2000; Asojo et al., 2005), XCRISP with hatching gland cells (Schambony et al., 2003), allurin with sperm (Olson et al., 2001). However, the molecular mechanisms involved in these interactions remain unknown. The results of the present work open the possibility that the conserved Signature 2 region plays a similar role in the function of other proteins of the family known to interact with cell membranes in different biological systems.

In conclusion, the interesting finding that the egg-binding ability of DE resides in an evolutionarily conserved region of the molecule provides novel information on the molecular mechanisms underlying the role of CRISP-1 proteins in mammalian gamete fusion with potential implications on the structure–function relationship of other members of a widely distributed family of proteins.

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