BIOLOGY OF REPRODUCTION **77,** 848–854 (2007)
Published online before print 1 August 2007.
DOI 10.1095/biolreprod.107.061788

A Novel Function for CRISP1 in Rodent Fertilization: Involvement in Sperm-Zona Pellucida Interaction[1]

Dolores Busso,[3,4] Débora J. Cohen,[3] Julieta A. Maldera, Andrea Dematteis, and Patricia S. Cuasnicu[2]

Instituto de Biología y Medicina Experimental (IBYME-CONICET), 1428 Buenos Aires, Argentina

ABSTRACT

Epididymal protein CRISP1 participates in rat and mouse gamete fusion through its interaction with complementary sites on the egg surface. Based on in vivo observations, in the present study we investigated the possibility that CRISP1 plays an additional role in the sperm-zona pellucida (ZP) interaction that precedes gamete fusion. In vitro fertilization experiments using zona-intact rat and mouse eggs indicated that the presence of either an antibody against rat CRISP1 (anti-CRISP1) or rat native CRISP1 (rCRISP1) during gamete co-incubation produced a significant decrease in the percentage of fertilized eggs. However, differently to that expected for a protein involved in gamete fusion, no accumulation of perivitelline sperm was observed, suggesting that the inhibitions occurred at the sperm-ZP interaction level. Bacterially expressed recombinant CRISP1 (recCRISP1) also significantly inhibited egg fertilization. In this case, however, an increase in the number of perivitelline sperm was observed. Subsequent experiments evaluating the effect of anti-CRISP1 or rCRISP1 on the number of sperm bound per egg indicated that the protein is involved in the initial step of sperm-ZP binding. In agreement with these functional studies, indirect immunofluorescence experiments revealed that although rCRISP1 is capable of binding to both the ZP and the oolema, recCRISP1 only binds to the egg surface. The finding that deglycosylated rCRISP1 behaves as the untreated protein, whereas the heat-denatured rCRISP1 associated only with the oolema, indicates that the protein ZP-binding ability resides in the conformation rather than in the glycosydic portion of the molecule. The interaction between rCRISP1 and the ZP reproduces the sperm-ZP-binding behavior, as judged by the failure of the protein to interact with the ZP of fertilized eggs. Together, these results support the idea that CRISP1 participates not only in sperm-egg fusion but also in the prior stage of sperm-ZP interaction.

epididymis, fertilization, gamete biology, ovum, sperm

INTRODUCTION

Mammalian fertilization involves cell-to-matrix (sperm-cumulus oophorus and sperm-zona pellucida [ZP]) and cell-to-cell (sperm and egg plasma membranes) interactions mediated by molecules present in both gametes. Although several candidate molecules have been proposed to participate in each of these events, results from genetic approaches showing the absence of a clear phenotype in knockout mice suggest that there must be other as-yet unidentified gamete components contributing to the fertilization process. For many years, our laboratory has been studying the participation of rat epididymal protein DE [1] in sperm-egg fusion. DE is also known as CRISP1 for being the first described member of the CRISP (Cysteine-RIch Secretory Proteins) family, which includes proteins widely distributed in animals, plants, and fungi [2–4]. Rat CRISP1 (rCRISP1) is a 32-kDa androgen-dependent protein with 10% of carbohydrates [5, 6] that associate with the sperm surface during epididymal transit [7, 8]. Originally localized in the dorsal region of the sperm head, rCRISP1 migrates to the equatorial segment as the acrosome reaction occurs [9], and it mediates gamete fusion through complementary sites on the egg surface [10]. 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 [10, 11], a region through which fusion rarely occurs [12, 13]. Structure-function studies using bacterially expressed rat CRISP1 (recCRISP1) revealed that the egg-binding ability of the protein does not involve the carbohydrate moieties and resides in the polypeptidic region of the molecule [14]. Further experiments using deletion mutants of the recombinant protein as well as synthetic peptides indicated that the active site of rCRISP1 resides in a 12-amino acid region corresponding to a highly conserved motif of the CRISP family [15]. Rat CRISP1 exhibits high homology with two other epididymal proteins known as mouse AEG-1/CRISP1 [16, 17] and human ARP/hCRISP1 [18, 19], both of which are also involved in gamete fusion through their interaction with binding sites on the surface of the corresponding eggs [20, 21].

While these findings support a role for CRISP1 in gamete fusion, circumstantial observations from our laboratory suggest that this protein might play an additional role in fertilization. The evidence for this hypothesis comes from experiments showing that sperm exposed to immune sera against rCRISP1 prior to uterine insemination fertilized a significantly lower percentage of eggs in the oviduct compared with controls [22]. Interestingly, the nonfertilized eggs did not exhibit sperm in the perivitelline space, as would be expected for the blocking of a protein involved in gamete fusion (Perez, unpublished observations), opening up the possibility that the anti-CRISP1 antibodies had interfered with a step of gamete interaction that precedes sperm-egg fusion.

In view of these in vivo observations, in the present work we have explored specifically the participation of CRISP1 in the stage of sperm interaction with the ZP. Results obtained in

[1]Supported by the World Health Organization (WHO) LID grant (H9/181/R429) to P.S.C. D.B. was a recipient of a fellowship from the National Research Council of Argentina (CONICET). J.A.M. is a recipient of a fellowship from CONICET. A.D. was a recipient of a fellowship from the Latinomerican Programme for Training and Research in Human Reproduction (PLACIRH).
[2]Correspondence: Patricia S. Cuasnicu, Instituto de Biología y Medicina Experimental, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina. FAX: 11 4786 2564; e-mail: cuasnicu@dna.uba.ar
[3]These authors contributed equally to this work.
[4]Current address: Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Avda Bernardo O'Higgins 340, 6513492 Santiago, Chile.

Received: 22 March 2007.
First decision: 29 April 2007.
Accepted: 30 July 2007.

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

both the rat and mouse models support the involvement of the protein in the first step of sperm binding to the ZP, identifying a novel role for CRISP1 during the fertilization process.

MATERIALS AND METHODS

Animals

Adult (90–120 days) male and immature (25–30 days) female Sprague-Dawley rats and adult (60–120 days) male and young adult (30–60 days) female CF1 mice were used for the experiments described in this work. All animals were bred in-house and 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 Institutes of Health (NIH).

Sperm Collection and In Vitro Capacitation

Rat sperm were recovered by squeezing the decapsulated cauda tubule section more proximal to the vas deferens into 0.6 ml Toyoda and Chang [23] fertilization medium supplemented with 0.4% BSA (Sigma Chemical Co., St. Louis, MO) under paraffin oil. After dispersion, sperm concentration was adjusted to 1.25×10^6 sperm/ml and the cells were capacitated during gamete co-incubation (see *In Vitro Fertilization*). Mouse sperm were recovered by incising cauda epididymal tubules in 0.3 ml of mouse Whittingham medium [24] containing 0.3% BSA under paraffin oil. Aliquots of the original suspension were diluted in 0.3 ml fresh capacitation medium—adjusting the final concentration to 0.1 to 1×10^7 cells/ml—and incubated for 90–120 min under paraffin oil at 37°C in an atmosphere of 5% CO_2 in air.

Recovery and Treatment of the Eggs

Female rats were stimulated to ovulate following the method described by Walton et al. [25]. Briefly, immature females weighting more than 60 g were injected i.p. with 5 IU eCG (Syntex, Buenos Aires, Argentina) 2–3 h after the beginning of the light period of Day −3. The day of the experiment (Day 0), oviducts were recovered and cumulus cells were dispersed by treating the egg-cumulus complexes with 0.1% hyaluronidase (type IV; Sigma). An average of 10 eggs per female were recovered. After washing, the eggs were placed in 50-μl drops of rat fertilization medium under oil for subsequent insemination. Mouse females were superovulated by an i.p. injection of 5 IU eCG, followed by the i.p. administration of 5 IU hCG (Sigma) 48 h later, and eggs were recovered from the oviducts 12–15 h after hCG injection. Cumulus cells were removed with 0.03% hyaluronidase, and after extensive washing, zona-intact eggs were placed in 100-μl drops of Whittingham medium under oil. A total of 10–15 eggs were used per treatment in each experiment.

In Vitro Fertilization (IVF)

Zona-intact rat eggs were inseminated with rat sperm prepared as previously described (final sperm concentration: 5×10^5 sperm/ml), and gametes were co-incubated at 37°C and 5% CO_2 for 24 h. Mouse eggs were inseminated with capacitated sperm at a final concentration of 0.1 to 1×10^6 cells/ml, and gametes were co-incubated for 7 h under the conditions described above. In both cases, at the end of the incubation, eggs were observed under a phase-contrast microscope (Nikon Optiphot, Nikon, Japan), and both the percentages of fertilized cells and the mean number of perivitelline sperm per egg were calculated. Eggs were scored as fertilized when two pronuclei and a sperm tail were observed in the ooplasm.

For experiments involving the effect of a polyclonal antibody against rCRISP1 (anti-CRISP1) on fertilization, sperm were incubated for 30 min in the presence or absence of the antibody (7.2 mg/ml) [26], or in the presence of normal rabbit serum or normal rabbit immunoglobulin (IgG; Sigma) prior to the addition of the eggs to the IVF drops. For those cases in which the effect of rCRISP1 on fertilization was examined, eggs were incubated for 30 min in medium alone or in medium containing 6 μM purified rCRISP1 [5, 6]; ovalbumin (OA; Sigma); recCRISP1, expressed as a fusion protein coupled to maltose-binding protein (MBP) [14]; or MBP prior to insemination.

Sperm-ZP Binding

Zona-intact mouse eggs were inseminated with capacitated sperm as previously described, and gametes were co-incubated for 30 min at 37°C and 5% CO_2. Eggs were then removed and washed by transfer through three drops of fresh medium using a wide-bored pipette to remove loosely associated sperm. Eggs were then fixed in 2% paraformaldehyde in PBS and mounted on slides, and the number of sperm bound to the ZP was scored under phase-contrast microscopy at 400× magnification.

Indirect Immunofluorescence

Zona-intact nonfertilized and/or in vitro-fertilized eggs were incubated in medium alone or medium containing the specified concentrations of either rCRISP1 (untreated, deglycosylated, or heat-denatured), recCRISP1, or MBP. Deglycosylation of rCRISP1 was carried out following the procedure described by Ellerman et al. [14]. Native rCRISP1 was denatured by heating the protein at 100°C for 10 min. In all the cases, the eggs were fixed for 1 h in 0.1 ml of 2% paraformaldehyde in PBS at room temperature and washed several times with PBS containing 4 mg/ml BSA (PBS-BSA4). Eggs were then incubated for 30 min at 37°C in 100 μl of 5% normal goat serum (NGS) in PBS-BSA4, and were exposed to anti-CRISP1 (1:50 in 1% NGS in PBS-BSA4) or anti-MBP (1:100 in 1% NGS in PBS-BSA4; New England BioLabs, Beverly, MA) for 1 h at 37°C. After washing in PBS-BSA4, eggs were incubated for 30 min at 37°C in fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:50 in PBS-BSA4; Sigma), washed, and finally mounted in 90% glycerol in PBS. Eggs were examined with a Nikon Optiphot microscope equipped with epifluorescence optics (250×).

Statistical Analysis

Results were expressed as mean values ± SEM for each series of experiments. Statistical significance of the data was analyzed using the chi-square test for the percentages of fertilized eggs. The numbers of perivitelline sperm per egg and bound sperm per egg were analyzed using the Student *t*-test. In all cases, results were considered significant at $P < 0.05$.

RESULTS

Participation of CRISP1 in Sperm-ZP Interaction

As a first approach to investigate the potential involvement of CRISP1 in sperm-ZP interaction, we evaluated the effect of a polyclonal antibody against rCRISP1 (anti-CRISP1) on in vitro fertilization. For this purpose, zona-intact rat eggs were added to drops containing sperm preincubated for 30 min in medium containing either anti-CRISP1 or normal rabbit serum. Results showed that the presence of the antibody during gamete co-incubation produced a significant decrease in the percentage of fertilized eggs compared with controls (Fig. 1A), with no presence of sperm in the perivitelline space. As previously reported [27], sperm agglutination or alterations in sperm motility were not observed during the incubation (data not shown), indicating that the inhibition in fertilization was not due to a deleterious effect of the antibody. As a second approach to the same question, the effect of purified rCRISP1 on fertilization was evaluated with the premise that if the sperm protein interacts with the ZP during fertilization, exposure of the eggs to rCRISP1 should competitively inhibit gamete interaction. In this case, zona-intact rat eggs were co-incubated with sperm in a medium containing either OA (as a control) or rCRISP1 at a concentration known to inhibit sperm fusion ability without affecting other sperm functional parameters [10]. Results revealed that rCRISP1 produced a significant decrease in the percentage of fertilized eggs compared with controls (Fig. 1B), with no presence of perivitelline sperm. Together, these results suggest an inhibitory effect of both the antibody and the protein at a step prior to sperm-egg fusion.

Since the conditions used in our studies to achieve rat IVF involved the use of fresh epididymal sperm that undergo capacitation during the gamete co-incubation period, the possibility existed that the inhibitions produced by either the antibody or the protein were due to an indirect inhibitory effect on sperm capacitation. In view of this, the effect of both the antibody and the protein on sperm-ZP interaction was reevaluated using the mouse IVF technique, in which the eggs are exposed to sperm that have already undergone capacitation.

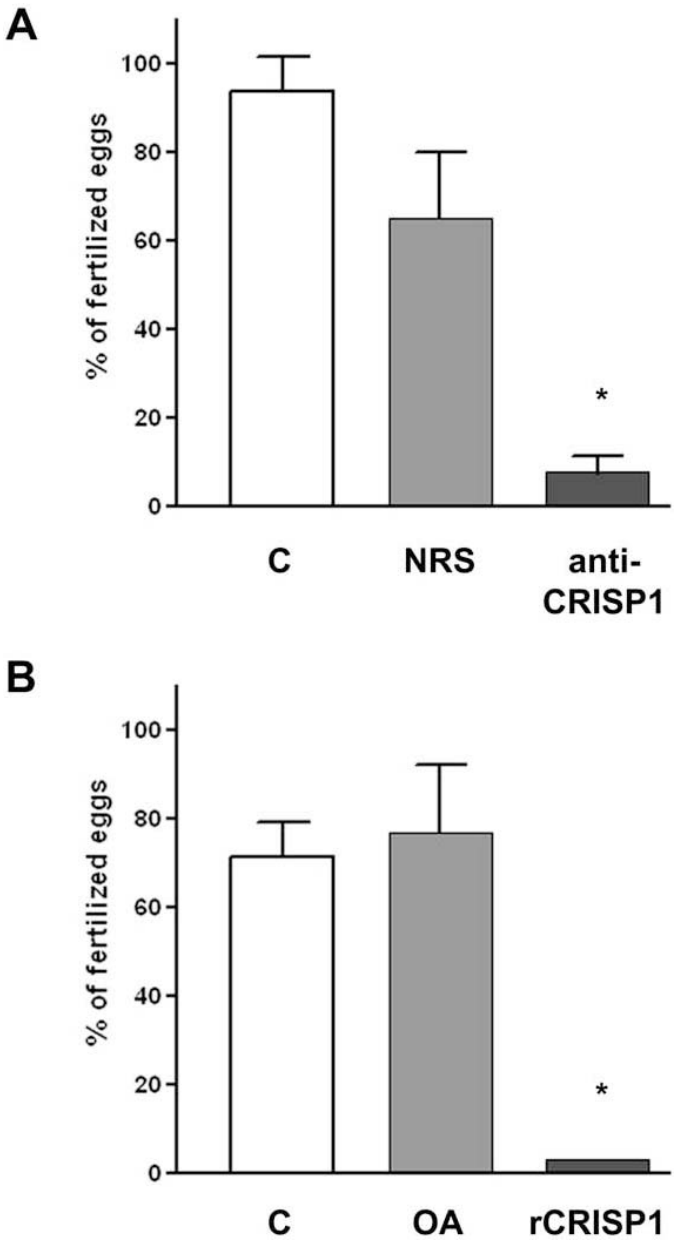

FIG. 1. Effect of anti-CRISP1 and rCRISP1 on rat IVF. **A**) ZP-intact eggs were inseminated with sperm preincubated in medium alone (C) or in medium containing either normal rabbit serum (NRS) or anti-CRISP1. **B**) ZP-intact eggs were preincubated in medium alone or containing 6 μmol/L of either OA or purified rCRISP1, and then were inseminated with epididymal sperm. At the end of gamete co-incubation, the percentages of fertilized eggs were determined. Number of experiments: n = 4 for **A**; n = 5 for **B**. *$P < 0.001$ versus controls (C, NRS, OA).

Co-incubation of zona-intact mouse eggs and capacitated sperm in the presence of anti-CRISP1 produced a significant decrease in the percentage of fertilized eggs compared with controls (Fig. 2A). As for the rat, sperm agglutination or alterations in sperm motility were not observed in the presence of the antibody (control: 45% ± 6%; anti-CRISP1: 47% ± 3%; IgG: 43% ± 2%).

For those experiments aiming to examine the effect of rCRISP1 on mouse gamete interaction, zona-intact eggs were incubated for 30 min in medium alone or medium containing 6 μM rCRISP1, and they then were inseminated with capacitated sperm. In parallel, mouse gametes were also exposed to the bacterially expressed recombinant CRISP1 (recCRISP1) or

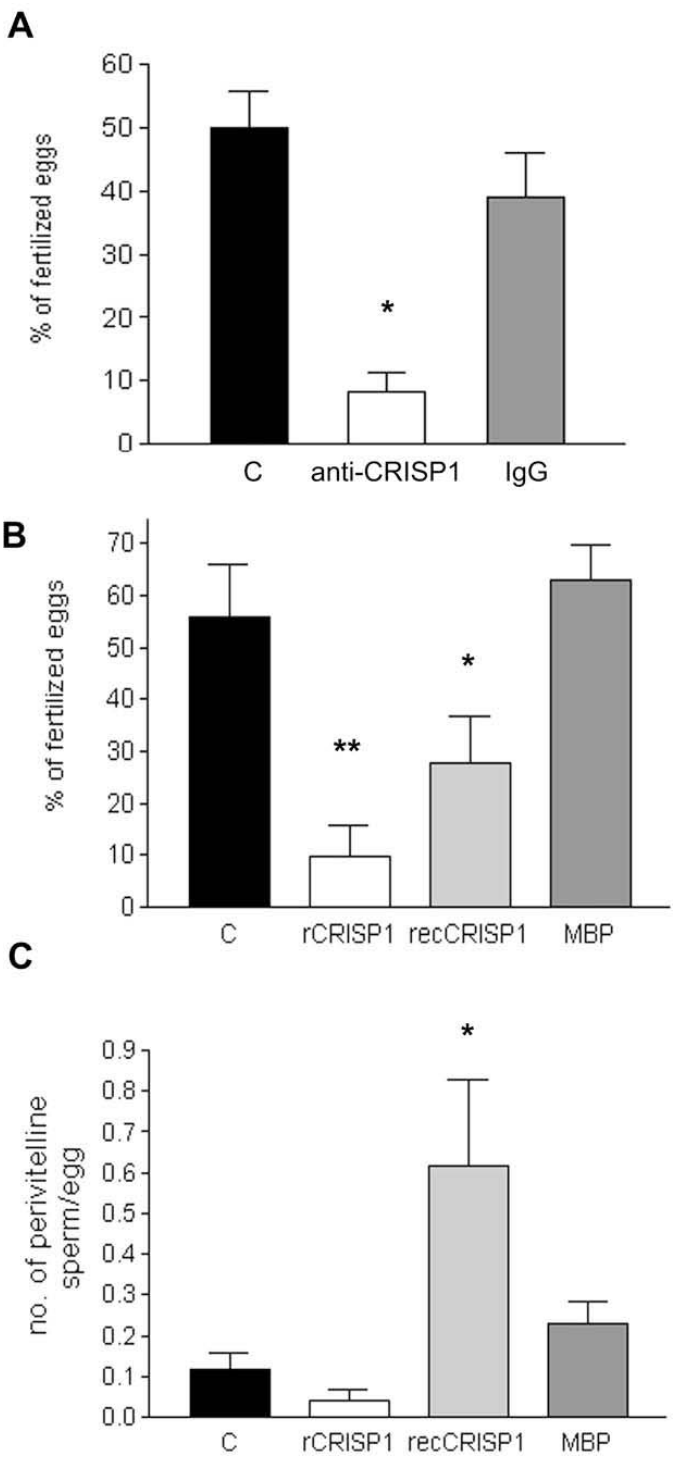

FIG. 2. Effect of anti-CRISP1 and rCRISP1 on mouse IVF. **A**) ZP-intact eggs were inseminated with capacitated mouse sperm in medium alone (C) or in medium containing normal rabbit IgG or anti-CRISP1, and the percentage of fertilized eggs was evaluated after gamete co-incubation. Number of experiments: n = 7; *$P < 0.05$ versus controls (C and IgG). **B**, **C**) ZP-intact eggs were preincubated in medium alone or containing 6 μmol/L of either purified rCRISP1, recCRISP1, or MBP, and they then were inseminated with capacitated mouse sperm. At the end of gamete co-incubation, the percentage of fertilized eggs (**B**) (**$P < 0.01$ versus controls [C, MBP], *$P < 0.05$ versus controls; rCRISP1 versus recCRISP1 NS) and the number of perivitelline sperm per egg (**C**) (*$P < 0.05$ versus C, rCRISP, and MBP) were determined (n = 10).

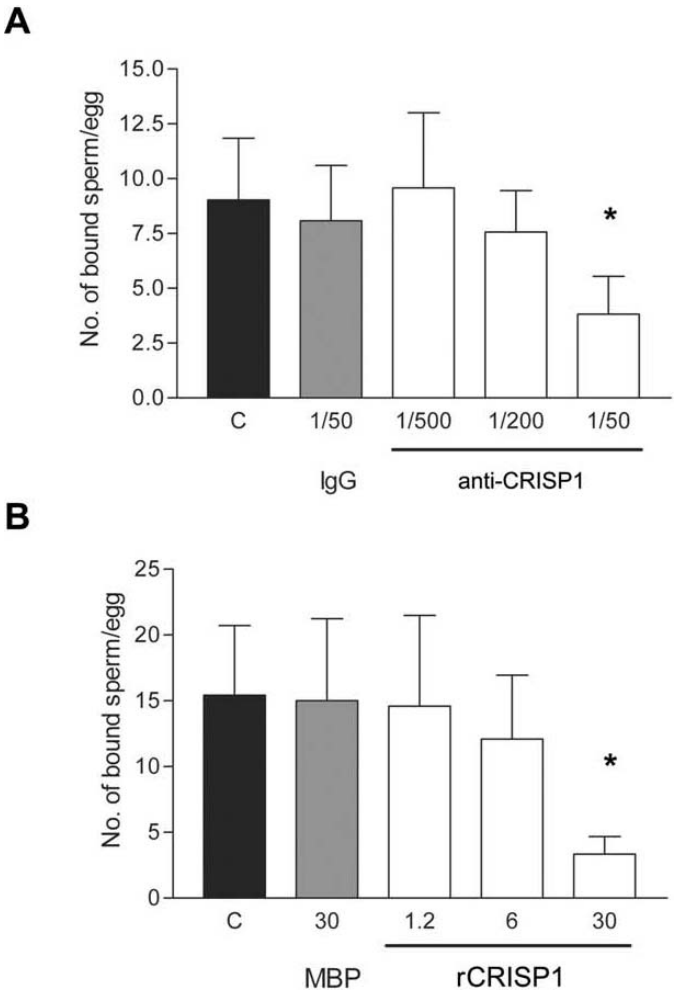

FIG. 3. Effect of anti-CRISP1 and rCRISP1 on mouse sperm-ZP binding. **A**) ZP-intact eggs were inseminated with capacitated mouse sperm preincubated in medium alone (C) or in medium containing either normal rabbit IgG or different dilutions of anti-CRISP1. **B**) ZP-intact eggs were preincubated in medium alone (C) or containing 30 μM MBP or different concentrations of purified rCRISP1, and they then were inseminated with capacitated mouse sperm. The number of bound sperm per egg was scored after 30 min of gamete co-incubation. Number of experiments: n = 3 for **A**; n = 8 for **B**; $*P < 0.05$.

MBP (control) as an attempt to investigate the relationship between the structure and function of the protein. Results showed that although the presence of either rCRISP1 or recCRISP1 significantly inhibited fertilization compared with controls (Fig. 2B), eggs incubated with recCRISP1 presented a significant increase in the number of perivitelline sperm per egg compared with rCRISP1 (Fig. 2C), indicating that the inhibitory effect of the recombinant protein was at the sperm-egg fusion level. Together, these results indicate that native rCRISP1, but not recCRISP1, would be interfering with the sperm-ZP interaction that precedes gamete fusion.

Considering that sperm-ZP interaction involves a first step of sperm binding to the zona, experiments were performed to examine the participation of CRISP1 in this specific event of fertilization. Zona-intact mouse eggs were inseminated with capacitated sperm in the presence of either anti-CRISP1 or rCRISP1, and the number of sperm bound to the ZP were evaluated after 30 min of gamete interaction. In both cases, results showed a significant and dose-dependent inhibition in the number of sperm bound per egg compared with the corresponding controls (Fig. 3, A and B).

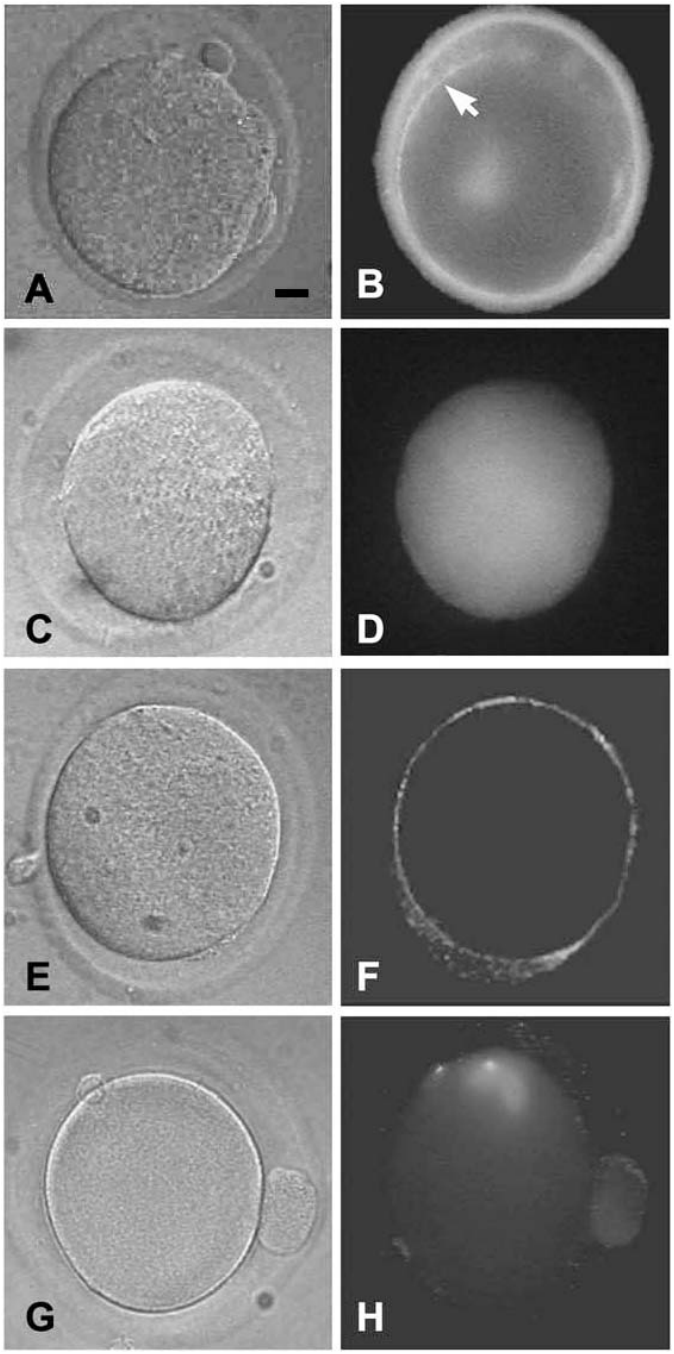

FIG. 4. Binding of rCRISP1 and recCRISP1 to zona-intact mouse eggs. Phase and fluorescent images of ZP-intact eggs incubated with 6 μmol/L of either purified rCRISP1 (**A**, **B**) or recCRISP1 (**E**, **F**), fixed, and subjected to IIF using anti-CRISP1 and anti-MBP as primary antibodies, respectively, were studied. Eggs incubated in medium alone (**C**, **D**) or 6 μM MBP (**G**, **H**) were used as controls. Note the fluorescent labeling in the ZP and oolema (arrow) in **B**, and the absence of labeling in the ZP in **F**. Original magnification ×250. Eggs incubated with deglycosylated rCRISP1 or heat-denatured rCRISP1 exhibited the same fluorescent patterns as those shown in **B** and **F**, repectively. Scale bar = 10 μm.

Interaction of CRISP1 with the ZP

The results described above support the involvement of CRISP1 in the initial step of sperm binding to the ZP through an interaction of the protein with the egg coat. To further investigate this possibility, zona-intact mouse eggs were incubated in medium containing 6 μmol/L of either rCRISP1,

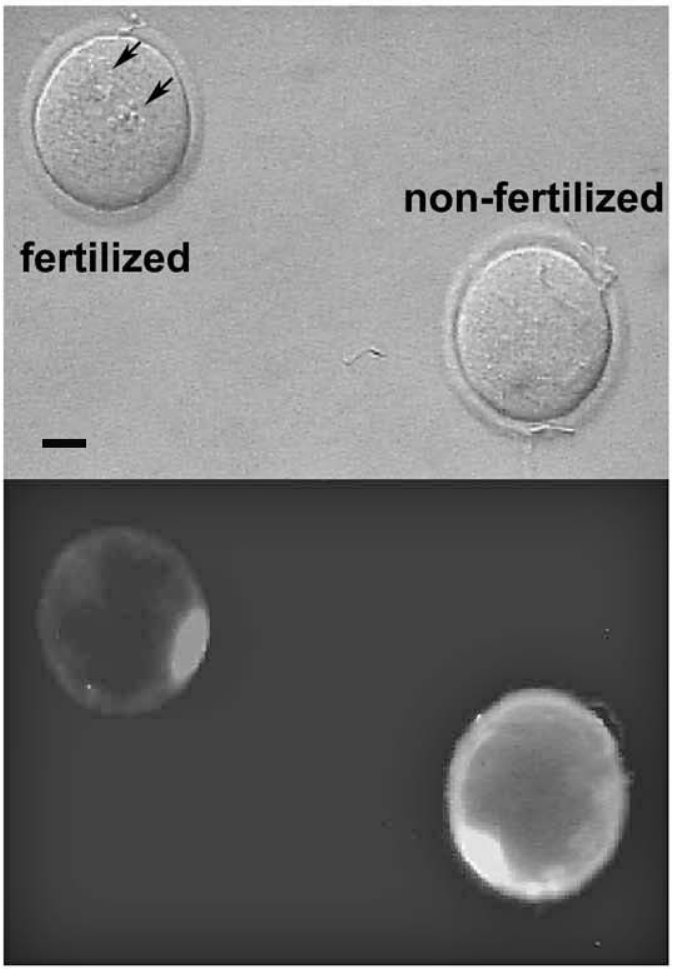

FIG. 5. Effect of fertilization on the binding of rCRISP1 to the ZP. Phase and fluorescent images of in vitro-fertilized and nonfertilized mouse eggs incubated in the same drop with rCRISP1, fixed, and subjected to IIF using anti-CRISP1 as primary antibody were studied. The arrows point to the pronuclei in the fertilized egg (upper panel). Note the fluorescent staining of ZP in the nonfertilized egg (lower panel). Original magnification ×150. Scale bar = 20 μm.

recCRISP1, or MBP, were fixed, and were subjected to IIF using anti-CRISP1 or anti-MBP as primary antibody. As shown in Figure 4, A–D, eggs incubated with rCRISP1 presented an intense and specific fluorescent labeling on the ZP, in addition to the reported labeling on the oolema [20]. Eggs incubated with recCRISP1, on the other hand, exhibited only a fluorescent labeling on the oolema (Fig. 4, E and F) not detected in controls (Fig. 4, G and H). The same fluorescence pattern was observed when the eggs were incubated with higher concentrations (15 and 30 μmol/L) of recCRISP1 (data not shown).

The failure of recCRISP1 to interact with the ZP suggests that carbohydrates and/or the protein conformation would be important for the association of rCRISP1 with the ZP. To further investigate these possibilities, rCRISP1 was either treated with PNGase F to remove the entire glycosydic core or was denatured by heating, and it then was evaluated by IIF for its ability to interact with the egg coat. Results revealed that although the deglycosylated protein was as capable as the untreated protein of associating with the ZP, the denatured protein became unable to bind to the ZP, retaining its ability to interact with the oolema (Fig. 4).

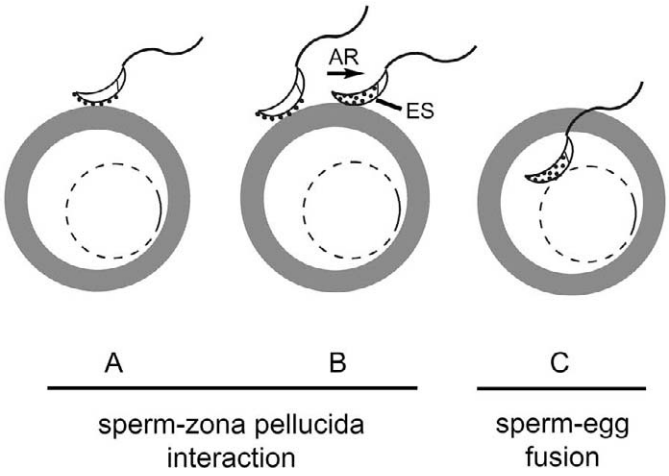

FIG. 6. Model for the participation of CRISP1 in the different steps of sperm-egg interaction. **A**) CRISP1 localized in the dorsal region of the sperm head participates in the binding of intact sperm to the ZP. **B**) The ZP induces the sperm acrosome reaction (AR), which triggers the migration of CRISP1 to the equatorial segment (ES) **C**) CRISP1, localized in the equatorial segment, mediates gamete fusion through the interaction between the Signature 2 region of the molecule and the complementary sites on the egg surface.

To control the specificity of the interaction between rCRISP1 and the ZP, IIF experiments were performed using fertilized zona-intact eggs, which exhibit a modified ZP as an adaptation to avoid polyspermy. For this purpose, nonfertilized and in vitro-fertilized eggs were incubated within the same drop with rCRISP1, and then were fixed and subjected to IIF using anti-CRISP1 as primary antibody. Results showed that although control nonfertilized eggs presented the intense ZP fluorescence as previously described in this report, the fertilized eggs exhibited only a very faint labeling on their ZP (Fig. 5).

DISCUSSION

Evidence from our laboratory strongly supports the participation of epididymal CRISP1 in gamete fusion through its binding to complementary sites on the egg surface. In vivo observations showing that rat sperm exposed to a serum against rCRISP1 prior to uterine insemination exhibited a significantly reduced fertilizing ability but did not accumulate in the perivitelline space, and this opened up the possibility for an additional role of CRISP1 during fertilization. In the present work, we provide evidence supporting the participation of CRISP1 in the sperm-ZP interaction event that precedes gamete fusion.

The first series of experiments involved the in vitro co-incubation of zona-intact rat eggs and rat sperm in the presence of the anti-CRISP1 antibody. The finding that there was a significant inhibition in egg fertilization with no accumulation of perivitelline sperm supports the involvement of the protein in sperm-ZP interaction. This was further supported by the significant decrease in the percentage of fertilized eggs observed in the presence of rCRISP1. Since the conditions required in our studies for obtaining a successful rat IVF involve the use of fresh sperm that undergo capacitation during gamete co-incubation, the effects of the antibody and the protein on fertilization were reevaluated using the mouse IVF technique in which the oocytes are co-incubated with capacitated sperm. In this regard, it is important to mention that anti-CRISP1 is able to specifically recognize the mouse

homolog on sperm [20] and that rCRISP1 is capable of binding to zona-free mouse eggs and inhibiting their penetration [20]. The results obtained in the mouse also showed a significant decrease in the percentage of fertilized eggs in the presence of either the antibody or the protein, again with no accumulation of perivitelline sperm. In this case, however, the insemination of eggs with sperm that have already been capacitated supports a specific inhibitory effect on sperm-egg interaction rather than on sperm capacitation. Moreover, although it has been reported that rCRISP1 is capable of inhibiting protein tyrosine phosphorylation when added to capacitating rat sperm [28], recent results suggest that the protein would not affect this event in mouse spermatozoa [29]. According to the authors, the reason for this difference may reflect species-specific differences in the regulation of the events associated with completion of sperm capacitation.

As stated before, previous results from our laboratory showed that recCRISP1, as native rCRISP1, has the ability to bind to the oolema and inhibit zona-free egg penetration [14]. The results obtained in the present study showed that recCRISP1 is also capable of inhibiting fertilization of zona-intact mouse eggs in vitro. However, in contrast to native rCRISP1, the presence of recCRISP1 produced an increase in the number of perivitelline sperm per egg, indicating that the recombinant protein interferes with the sperm-oolema interaction. This conclusion is further supported by IIF results showing that although eggs incubated with rCRISP1 exhibited fluorescent labeling in both the ZP and the oolema, those exposed to recCRISP1 were labeled only on the oolema. Thus, although recCRISP1 behaves as native rCRISP1 regarding its interaction with the oolema, it does not mimic the behavior of the native protein at the ZP level.

Considering the lack of glycosylation in bacterially expressed proteins, it is possible that carbohydrates played a role in the interaction between rCRISP1 and the ZP. Indirect immunofluorescence results revealed, however, that deglycosylated rCRISP1 was as capable as the untreated protein of interacting with the ZP, suggesting that the protein conformation would be important for its association with the ZP. This conclusion was further supported by the finding that the heat-denatured rCRISP became unable to associate with the ZP while retaining its ability to interact with the oolema. Together, these findings indicate that the ability of CRISP1 to interact with the ZP resides in the conformation rather than in the glycosydic portion of the molecule. By contrast, neither the carbohydrates nor the conformation is essential for the interaction of CRISP1 with the oolema [14]. Furthermore, recent results reveal that the egg-binding ability of CRISP1 resides in a 12-amino acid region corresponding to one of the evolutionarily conserved motifs of the CRISP family known as Signature 2 [15].

The results showing that both anti-CRISP1 and rCRISP1 significantly inhibited the number of mouse sperm bound per egg supports the participation of CRISP1 in the first step of sperm binding to the ZP. In this regard, the observation that the fluorescent labeling on the ZP of rCRISP1-incubated eggs was almost undetectable in fertilized eggs suggests that the ability of the ZP components to bind rCRISP1 must have been modified as a consequence of the reactions involved in the block to polyspermy. The finding that the interaction between rCRISP1 and the ZP reproduces that observed between the sperm and the ZP before and after fertilization supports the physiological relevance of our observations.

The molecular basis of sperm-ZP binding remains controversial. Several sperm components have been proposed to mediate this interaction, suggesting the existence of protein redundancy as a mechanism to ensure the association of capacitated sperm with the egg coat. Surface sperm proteins, such as galactosyltranferase and SED1, have been proposed to participate in the initial adhesion of intact sperm to the ZP [30], whereas intra-acrosomal proteins, exposed after the acrosome reaction, such as sp56 and zonadhesin, have been proposed to mediate the binding of reacted sperm to the egg coat [31–34]. According to this model, the localization of CRISP1 on the surface of intact sperm supports its participation in the initial sperm-ZP binding step. Although the species specificity of fertilization mostly resides at the sperm-ZP interaction level, the first step of sperm-ZP binding seems to be less species specific than the ZP penetration event [35, 36]. In this regard, a recent report describes rat sperm as able to bind to the mouse ZP and suggests that the components required for sperm-ZP binding must be conserved between these two species [37]. Our results showing that rCRISP1 is able to interact with the mouse ZP and inhibit mouse sperm-ZP binding are in agreement with these observations.

The present work, together with our previous results, in both the rat and the mouse [9, 10, 38] led us to propose a model for the participation of CRISP1 in fertilization (Fig. 6). CRISP1, localized on the dorsal region of capacitated intact sperm, participates in the first step of sperm binding to the ZP through a conformation-mediated mechanism. Then, the ZP induces the acrosome reaction, which triggers the migration of CRISP1 to the equatorial segment. Once localized in the fusogenic region of the sperm head, CRISP1 mediates gamete fusion through the interaction between the Signature 2 region and the complementary sites on the egg surface [15].

Increasing evidence supports the involvement of sperm proteins in more than one step of the fertilization process. Some examples of multifunctional proteins are as follows: acrosin—proposed to participate in the release of proteins from the acrosomal matrix, binding of acrosome-reacted sperm to and penetration of the ZP, and the acquisition of fusogenicity of the equatorial segment after the acrosome reaction [39, 40]; PH-20—proposed to participate in cumulus penetration and binding to ZP2 [41, 42]; and galactosyltransferase—proposed to hydrolyze the glycosylic residues of the ZP and to mediate both binding to ZP3 and ZP3-induced acrosome reaction [43, 44]. According to our observations, CRISP1 is another example of a protein playing multiple roles during sperm-egg interaction. These results, together with our recent finding that testicular CRISP2 is also involved in sperm-egg fusion [45, 46], support the idea of different functional roles for the same CRISP and the involvement of different CRISPs in the same fertilization event. Considering that sperm are transcriptionally inactive cells, it is possible that this functional redundancy of proteins in sperm has evolved as a mechanism to ensure the success of the fertilization process.

ACKNOWLEDGMENT

The authors thank Dr. Diego A. Ellerman for constructive comments on the manuscript.

REFERENCES

1. Cameo MS, Blaquier JA. Androgen-controlled specific proteins in rat epididymis. J Endocr 1976; 69:317–324.
2. Fernandez C, Szyperski T, Bruyere T, Ramage P, Mosinger E, Wuthrich K. NMR solution structure of the pathogenesis-related protein P14a. J Mol Biol 1997; 266:576–593.
3. Henriksen A, King TP, Mirza O, Monsalve RI, Meno K, Ipsen H, Larsen JN, Gajhede M, Spangfort MD. Major venom allergen of yellow jackets, Ves v 5: structural characterization of a pathogenesis-related protein superfamily. Proteins 2001; 45:438–448.

4. Serrano RL, Kuhn A, Hendricks A, Helms JB, Sinning I, Groves MR. Structural analysis of the human Golgi-associated plant pathogenesis related protein GAPR-1 implicates dimerization as a regulatory mechanism. J Mol Biol 2004; 339:173–183.
5. Garberi JC, Fontana JD, Blaquier JA. Carbohydrate composition of specific rat epididymal protein. Int J Androl 1982; 5:619–626.
6. Garberi JC, Kohane AC, Cameo MS, Blaquier JA. Isolation and characterization of specific rat epididymal proteins. Mol Cell Endocrinol 1979; 13:73–82.
7. 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.
8. Kohane AC, Gonzalez Echeverria F, Piñeiro L, Blaquier JA. Interaction of proteins of epididymal origin with spermatozoa. Biol Reprod 1980; 23: 737–742.
9. 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.
10. 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.
11. 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.
12. 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.
13. Ebensperger C, Barros C. Changes at the hamster oocyte surface from germinal vesicle stage to ovulation. Gam Res 1984; 9:387–397.
14. Ellerman DA, Da Ros VG, Cohen DJ, Busso D, Morgenfeld MM, Cuasnicu PS. Expression and structure-function analysis of DE, a sperm cysteine-rich secretory protein that mediates gamete fusion. Biol Reprod 2002; 67:1225–1231.
15. Ellerman DA, Cohen DJ, Da Ros VG, Morgenfeld MM, Busso D, Cuasnicu PS. Sperm protein "DE" mediates gamete fusion through an evolutionarily conserved site of the CRISP family. Dev Biol 2006; 297: 228–237.
16. 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.
17. 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.
18. 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 assigment of the gene (AEGL1). Genomics 1996; 32:367–374.
19. 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.
20. 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.
21. 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.
22. 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.
23. Toyoda Y, Chang MC. Fertilization of rat eggs in vitro by epididymal spermatozoa and the development of eggs following transfer. J Reprod Fertil 1974; 36:9–22.
24. Fraser LR, Drury LM. The relationship between sperm concentration and fertilization in vitro of mouse eggs. Biol Reprod 1975; 13:513–518.
25. Walton E, Armstrong DT. Oocyte normality after superovulation in immature rats. J Reprod Fertil 1983; 67:309–314.
26. Kohane AC, Piñeiro L, Blaquier JA. Androgen-controlled synthesis of specific protein in the rat epididymis. Endocrinology 1983; 112:1590–1596.
27. 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.
28. Roberts KP, Wamstad JA, Ensrud KM, Hamilton DW. Inhibition of capacitation-associated tyrosine phosphorylation signaling in rat sperm by epididymal protein crisp-1. Biol Reprod 2003; 69:572–581.
29. Nixon B, MacIntyre DA, Mitchell LA, Gibbs GM, O'Bryan M, Aitken RJ. The identification of mouse sperm-surface-associated proteins and characterization of their ability to act as decapacitation factors. Biol Reprod 2006; 74:275–287.
30. Shur BD, Rodeheffer C, Ensslin MA, Lyng R, Raymond A. Identification of novel gamete receptors that mediate sperm adhesion to the egg coat. Mol Cell Endocrinol 2006; 250:137–148.
31. Cohen N, Wassarman PM. Association of egg zona pellucida glycoprotein mZP3 with sperm protein sp56 during fertilization in mice. Int J Dev Biol 2001; 45:569–576.
32. Kim KS, Cha MC, Gerton GL. Mouse sperm protein sp56 is a component of the acrosomal matrix. Biol Reprod 2001; 64:36–43.
33. Lea IA, Sivashanmugam P, O'Rand MG. Zonadhesin: characterization, localization, and zona pellucida binding. Biol Reprod 2001; 65:1691–1700.
34. Bi M, Hickox JR, Winfrey VP, Olson GE, Hardy DM. Processing, localization and binding activity of zonadhesin suggest a function in sperm adhesion to the zona pellucida during exocytosis of the acrosome. Biochem J 2003; 375:477–488.
35. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, 2nd ed. New York: Raven Press; 1994: 189–317.
36. Clark GF, Dell A. Molecular models for murine sperm-egg binding. J Biol Chem 2006; 281:13853–13856.
37. Boja ES, Hoodbhoy T, Garfield M, Fales HM. Structural conservation of mouse and rat zona pellucida glycoproteins. Probing the native rat zona pellucida proteome by mass spectrometry. Biochemistry 2005; 44:16445–16460.
38. 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.
39. Takano H, Yanagimachi R, Urch UA. Evidence that acrosin activity is important for the development of fusibility of mammalian spermatozoa with the oolemma: inhibitor studies using the golden hamster. Zygote 1993; 1:79–91.
40. Howes E, Pascall JC, Engel W, Jones R. Interactions between mouse ZP2 glycoprotein and proacrosin: a mechanism for secondary binding of sperm to the zona pellucida during fertilization. J Cell Sci 2001; 114:4127–4136.
41. Primakoff P, Hyatt H, Myles DG. A role for the migrating sperm surface antigen PH-20 in guinea pig sperm binding to the egg zona pellucida. J Cell Biol 1985; 101:2239–2244.
42. Lin Y, Mahan K, Lathrop WF, Myles DG, Primakoff P. A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. J Cell Biol 1994; 125:1157–1163.
43. Shur BD. Glycosyltransferases as cell adhesion molecules. Curr Opin Cell Biol 1993; 5:854–863.
44. Talbot P, Shur BD, Myles DG. Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion. Biol Reprod 2003; 68:1–9.
45. Busso D, Cohen DJ, Hayashi M, Kasahara M, Cuasnicu PS. Human testicular protein TPX1/CRISP-2: localization in spermatozoa, fate after capacitation and relevance for gamete interaction. Mol Hum Reprod 2005; 11:299–305.
46. Busso D, Goldweic NM, Hayashi M, Kasahara M, Cuasnicu PS. Evidence for the involvement of testicular protein CRISP2 in mouse sperm-egg fusion. Biol Reprod 2007; 76:701–708.