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Evidence for the Involvement of Testicular Protein CRISP2 in Mouse Sperm-Egg Fusion[1]

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ABSTRACT

CRISP2, originally known as Tpx-1, is a cysteine-rich secretory protein specifically expressed in male haploid germ cells. Although likely to be involved in gamete interaction, evidence for a functional role of CRISP2 in fertilization still remains poor. In the present study, we used a mouse model to examine the subcellular localization of CRISP2 in sperm and its involvement in the different stages of fertilization. Results from indirect immunofluorescence and protein extraction experiments indicated that mouse CRISP2 is an intraacrosomal component that remains associated with sperm after capacitation and the acrosome reaction (AR). In vitro fertilization assays using zona pellucida-intact mouse eggs showed that an antibody against the protein significantly decreased the percentage of penetrated eggs, with a coincident accumulation of perivitelline sperm. The failure to inhibit zona pellucida penetration excludes a detrimental effect of the antibody on sperm motility or the AR, supporting a specific participation of CRISP2 at the sperm-egg fusion step. In agreement with this evidence, recombinant mouse CRISP2 (recCRISP2) specifically bound to the fusogenic area of mouse eggs, as previously reported for rat CRISP1, an epididymal protein involved in gamete fusion. In vitro competition investigations showed that incubation of mouse zona-free eggs with a fixed concentration of recCRISP2 and increasing amounts of rat CRISP1 reduced the binding of recCRISP2 to the egg, suggesting that the proteins interact with common complementary sites on the egg surface. Our findings indicate that testicular CRISP2, as observed for epididymal CRISP1, is involved in sperm-egg fusion through its binding to complementary sites on the egg surface, supporting the idea of functional cooperation between homologous molecules to ensure the success of fertilization.

fertilization, ovum, sperm, testis

INTRODUCTION

Mouse protein Tpx-1, specifically expressed in haploid testicular germ cells [1, 2], is a secretory cysteine-rich molecule exhibiting high homology (69%) to rat epididymal protein DE [3, 4], first described by our laboratory [5]. It was this homology and the subsequent identification of several proteins showing significant similarity to Tpx-1 and DE [6–10] that confirmed the existence of a new protein family called cysteine-rich secretory protein (CRISP). As the first two members described, DE was named CRISP1, while Tpx-1 was named CRISP2. Since then, other members of the protein family have been identified such as CRISP3, which has a wide tissue distribution that includes salivary glands, human neutrophils, prostate, pancreas, pre-B cells, thymus, colon, and ovary [7, 11, 12], as well as CRISP4, a recently identified protein expressed in the epididymis [13, 14]. In addition to these mammalian CRISPs, members of the protein family have been identified in venoms from snails, snakes, and lizards [15–17] and in eggs and embryos from *Xenopus* [18, 19]. Moreover, molecular similarities between CRISPs and other proteins in less closely related organisms (i.e., allergens from insect venoms and pathogenesis-related proteins from plants [20, 21]) confirmed the existence of the cysteine-rich antigen 5 pathogenesis-related (CAP) protein superfamily.

Although numerous members of the CRISP family have been identified in recent years, few have well-characterized biological functions [15, 17, 22]. In the rat, studies of CRISP1 demonstrated that this protein (expressed in the epididymis under androgen regulation [23]) associates with the dorsal region of the acrosome during epididymal maturation [24, 25] and migrates to the equatorial segment as the acrosome reaction (AR) occurs [26]. The finding that exposure of zona-free eggs to CRISP1 significantly inhibits the percentage of penetrated eggs supported the involvement of this protein in gamete fusion through its binding to complementary sites localized on the egg surface [22, 27]. Subsequent studies revealed that homologous proteins for rat CRISP1 in mouse and human would also participate in sperm-egg fusion through their interaction with binding sites on the surface of the corresponding eggs [28, 29].

The analysis of the aminoacidic sequence of CRISP2 using various bioinformatic software shows that the protein contains a putative N-terminal peptide that supports its secretory nature and no hydrophobic regions or glycosylation or glycosyl phosphatidylinositol-anchoring sequences. Regarding functional evidence, CRISP2 was originally described as one of the primary autoantigens abundantly present within the guinea pig acrosome [30]. In rat sperm, CRISP2 was found to exist as a component of the acrosome and the outer dense fibers of the tail [10, 31], and it was recently proposed to play a role as a regulator of calcium influx through ryanodine receptors during capacitation [32]. Other groups reported the localization of CRISP2 on the surface of spermatogenic cells and postulated that the protein could be responsible for the interaction between

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germ cells and Sertoli cells [33, 34]. Recent results from our group showed that the human homologue of rodent CRISP2 would be an intraacrosomal component potentially relevant to gamete interaction, although its role in fertilization could not be confirmed because of the technical and ethical limitations relating to human gametes [35]. In the present study, we examined the subcellular localization and functional role of CRISP2 using a mouse model that allowed the analysis of the participation of the protein in the different stages of fertilization and the involvement of complementary sites on the egg surface in this process.

MATERIALS AND METHODS

Animals

Adult male (age, 60–120 days) and young adult female (age, 30–60 days) CF1 mice were maintained at 23°C with a 12 L:12D cycle. Experiments were conducted in accord with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction.

Antibodies

Rabbit polyclonal antibody against human recombinant CRISP2 (anti-CRISP2) (4.94 mg/ml) was raised by immunization of rabbits with the recombinant protein, followed by immunoglobulin G (IgG) purification, as described previously [3]. Anti-CRISP1 (7.2 mg/ml) was developed in our laboratory against purified rat CRISP1 [36], as previously reported [23].

Preparation of Tissue Cytosols and Sperm Extracts

Mouse testes were homogenized in 4 volumes of ice-cold 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiotreitol, 0.2% sodium azide, and 2 mM PMSF, using an Ultra-Turrax (IKA-WERK; Jauke & Hunkel, Staufen, Germany). The homogenates were then centrifuged for 20 min at 10 000 x *g* and 4°C, the supernatants were centrifuged at 100 000 x *g* for 1 h, and the cytosol fractions were stored at −20°C until use.

For sperm protein analysis, mouse cauda epididymal sperm were allowed to disperse in PBS at 37°C, were washed three times in PBS, and were then suspended in lysis buffer (150 mM NaCl , 50 mM Tris-HCl, and 1% Nonidet-P40) to obtain total sperm protein extracts or in the following extraction solutions: (1) PBS; (2) O.6 M NaCl in PBS; (3) citrate buffer (pH 4.5); (4) 100 mM Na_2CO_3 buffer (pH 11); (5) acrosomal matrix (AM) buffer (500 mM sodium acetate [pH 5.2] containing 0.11 M NaCl and 0.625% Triton X-100; Sigma, St. Louis, MO), reported to preserve the integrity of the AM [37]; and (6) 1% TX-114 and phase partitioning to analyze the lipophylic properties of the extracted proteins [38]. Protein extracts were concentrated by precipitation after addition of 1 volume of 10% ice-cold trichloroacetic acid, were suspended in Laemmli sample buffer [39] and were subjected to SDS-PAGE and Western blot analysis.

SDS-PAGE and Western Blot Analysis

Samples were separated by 10% SDS-PAGE under nonreducing conditions according to the method of Laemmli [39], and proteins were electrotransferred to nitrocellulose [40]. To ensure correct loading of proteins in each lane, the membranes were stained with Ponceau reagent (1 mg/ml of Ponceau S [Sigma] in 5% acetic acid) before immunodetection. The membranes were blocked for 1 h with powdered skim milk (2% in PBS) and were incubated for 1–2 h with anti-CRISP2 (dilution, 1:1000) or anti-CRISP1 (dilution, 1:200). Membranes were washed thoroughly and were incubated for 1 h with biotin-conjugated antirabbit IgG (1:500; Sigma). After extensive washing, the membranes were incubated for 1 h with ExtrAvidin-horseradish-peroxidase (1:1000; Sigma), and reactive bands were visualized with 3,3′-diaminobenzidine (40 μg/ml [Sigma] in 0.1 M Tris [Sigma] [pH 7.5] and 0.01% H_2O_2). All incubations were carried out at room temperature.

Indirect Immunofluorescence of Sperm

Mouse caudal epididymal sperm were fixed for 10 min in 2% paraformaldehyde in PBS at room temperature, extensively washed with PBS, and air dried on glass slides. For permeabilization, the slides were immersed in ice-cold methanol for 5 min before indirect immunofluorescence (IIF). Sperm were incubated in 5% normal goat serum in PBS (NGS-PBS) for 30 min at 37°C and were exposed to anti-CRISP2 (dilution, 1:100) in 1% NGS-PBS for 2 h at 37°C. After washing three times in PBS, sperm were incubated for 30 min at 37°C with fluorescein isothiocyanate (FITC)-IgG (1:100 in PBS), washed, and observed under an epifluorescence microscope. Normal rabbit IgG (5 mg/ml; Sigma) in 1% NGS-PBS was used as a negative control.

In Vitro Sperm Capacitation

Mouse sperm were recovered by incising cauda epididymal tubules in 0.3 ml of mouse Whittingham capacitation medium [41] supplemented with 0.3% BSA under paraffin oil. Aliquots of the original suspension were diluted in 0.3 ml of fresh capacitation medium, adjusting the final concentration to 0.1–1 × 10^7 cells/ml. Culture dishes (Falcon Plastics, Los Angeles, CA) containing 0.3-ml drops of the sperm suspension were incubated for 90–120 min under paraffin oil at 37°C in an atmosphere of 5% CO_2 in air.

Induction and Evaluation of the AR

For induction of the AR, ionophore A23187 (Sigma) was added (10 μM final concentration) to capacitated mouse sperm 30 min before the end of the capacitation period. The occurrence of the AR was evaluated by Coomassie brilliant blue staining. Sperm were fixed in 1 volume of 8% paraformaldehyde in PBS for 1 h at 4° C, washed with 0.1 M ammonium acetate (pH 9) by centrifugation, mounted on slides, and air dried. Slides were washed by successive immersions in water, methanol, and water (5 min each) and were then incubated in 0.22% Coomassie brilliant blue solution (50% methanol and 10% acetic acid). After staining, slides were thoroughly washed with distilled water, mounted, and immediately observed to avoid diffusion of the stain. Sperm were scored as acrosome intact when a bright blue staining was observed on the dorsal region of the acrosome or as acrosome reacted when staining was patchy or absent.

Recovery and Treatment of Mouse Oocytes

Females were superovulated by i.p. injection of 5 IU of eCG (Syntex, Buenos Aires, Argentina), followed by i.p. administration of 5 IU of hCG (Sigma) at 48–50 h later. Eggs were obtained from the oviducts of superovulated animals at 12–15 h after hCG administration. Cumulus cells were removed by incubating the oocyte-cumulus complexes for 3 min in capacitating medium containing 0.1% hyaluronidase (type IV; Sigma). For removal of the zonae pellucidae (ZP), eggs were treated with acid Tyrode solution (pH 2.5) for 10–20 seconds [42]. Zona-intact and zona-free eggs were thoroughly washed before their use in fertilization or immunofluorescence studies.

In Vitro Fertilization Assays

Mouse zona-intact eggs were inseminated with capacitated sperm (0.1–1 × 10^6 cells/ml), and gametes were coincubated at 37°C and 5% CO_2 for 7 h. After this period, eggs were washed in warm capacitating medium, mounted on slides, and observed under an optical microscope. The percentages of fertilized and ZP-penetrated eggs and the mean number of perivitelline sperm per egg were calculated for each treatment. Eggs were scored as fertilized when two pronuclei and a sperm tail were observed in the ooplasm. For experiments involving the effect of anti-CRISP2 on fertilization, sperm were incubated for 30 min in the presence of a 1:50 dilution of the antibody or normal rabbit IgG as a control before the addition of the eggs to the fertilization drops.

Expression of Recombinant Mouse CRISP2

A cDNA fragment corresponding to mouse CRISP2, previously isolated from a testis library [3], was ligated into the pMAL-C2 vector (New England BioLabs Inc., Beverly, MA) and was used to transform *Escherichia coli* bacteria. Recombinant mouse CRISP2 (recCRISP2) was expressed as a fusion protein coupled to maltose binding protein (MBP) by induction with isopropyl-1-thio-β-galactoside (IPTG) (Promega, Madison, WI). RecCRISP2 was then purified by affinity chromatography through an amylose (maltose polymer) resin column (New England BioLabs Inc.). The presence of recCRISP2 in bacterial extracts was evaluated by SDS-PAGE and Coomassie brilliant blue staining, and the purity of the protein was analyzed by SDS-PAGE and silver staining and Western blot.

IIF of Eggs

Mouse eggs were incubated for 1 h in medium alone or in medium containing 6 μM recCRISP2 or MBP, were fixed for 1 h in 1 ml of 2%

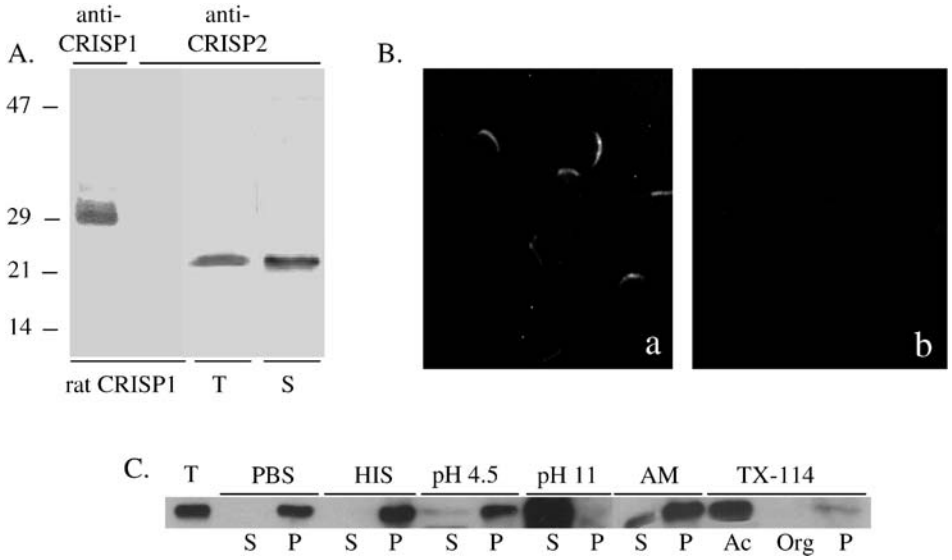

FIG. 1. Analysis of the presence of CRISP2 in mouse sperm. **A**) Western blot of purified rat CRISP1, mouse testicular cytosol (T), and sperm total protein extract (S) using anti-CRISP1 or anti-CRISP2 as first antibodies. **B**) Micrographs of mouse permeabilized epididymal sperm subjected to IIF using anti-CRISP2 (a) or normal rabbit IgG (b). Note the fluorescent staining over the dorsal region of the acrosome. Original magnification ×400. **C**) Western blot (using anti-CRISP2) of supernatants (S) and pellets (P) obtained after extraction of sperm proteins with PBS, high ionic strength buffer (HIS), citrate buffer (pH 4.5), carbonate buffer (pH 11), AM buffer, and TX-114. Aq and Org correspond to the aqueous and organic phases, respectively, after phase partitioning.

paraformaldehyde in PBS at room temperature, and were then washed several times with PBS containing 4 mg/ml of BSA (PBS-BSA4). Eggs were then incubated for 30 min at 37°C in 100 μl of 5% NGS in PBS-BSA4 and were then exposed to anti-MBP or normal rabbit IgG (1:100 in 1% NGS in PBS-BSA4) for 1 h at 37°C. After washing in PBS-BSA4, eggs were incubated for 30 min at 37°C in FITC-conjugated antirabbit IgG (1:50 in PBS-BSA4), were washed, and were finally mounted in 90% glycerol in PBS. Eggs were examined using an Optiphot microscope (Nikon, Tokyo, Japan) equipped with epifluorescence optics. For competition studies, zona-free eggs were incubated for 1 h in medium containing 6 μM recCRISP2 alone or together with increasing concentrations (0.6, 6, and 30 μM) of purified native rat epididymal CRISP1 [36, 43]. Eggs were then fixed and were subjected to IIF using anti-MBP or anti-CRISP1 as first antibodies.

Statistical Analysis

Results are expressed as mean values ± SEM for each series of experiments. Statistical significance of the data was analyzed using two-way ANOVA for the percentages of immunofluorescent patterns and sperm of different capacitation status and χ^2 test for the percentages of fertilized and ZP-penetrated eggs. The number of perivitelline sperm per egg was analyzed using Student *t*-test. In all cases, results were considered significant at a $P < 0.05$. The correlations between sperm with intact or reacted acrosomes and fluorescent patterns A or T were analyzed by Pearson product moment correlation. The tests were considered significant at $r > 0.95$.

RESULTS

Localization of CRISP2 in Sperm

As a first step toward understanding the function of CRISP2 during gamete interaction, we studied its localization in mouse sperm by IIF using anti-CRISP2 [35]. For this purpose, we first analyzed the ability of this antibody to recognize the mouse protein without crossreacting with the highly homologous epididymal protein CRISP1. Results showed that anti-CRISP2 did not crossreact with purified rat CRISP1 (32 kDa) and specifically recognized a unique band of approximately 25 kDa (expected molecular weight for CRISP2) in mouse testicular cytosol and sperm total protein extract (Fig. 1A). Having established the specificity of anti-CRISP2, we then localized CRISP2 in mouse sperm. While cells fixed in paraformaldehyde and then incubated with anti-CRISP2 showed faint fluorescent staining in the acrosomal region (data not shown), those permeabilized with cold methanol or fixed in membrane-permeabilizing acetone exhibited strong fluorescence in the dorsal region of the acrosome, sometimes accompanied by weak labeling in the principal piece of the tail (Fig. 1Ba). Sperm subjected to IIF using normal rabbit IgG instead of anti-CRISP2 were unstained (Fig. 1Bb). To analyze the subcellular localization of CRISP2, we next evaluated the behavior of the protein after a series of extraction procedures indicative of the kinds of association of CRISP2 with different sperm components. After the extractions, sperm suspensions were centrifuged, and the presence of CRISP2 in each of the supernatants and sperm pellets was determined by Western blot analysis. Results shown in Figure 1C revealed that neither PBS alone nor high ionic strength buffer (0.6 M NaCl) was able to remove CRISP2 from sperm. The two pH conditions used resulted in a differential extraction of CRISP2 from sperm, as most of the protein was removed by the high pH buffer, but it remained associated with the sperm pellet after incubation in the acidic solution. A high proportion of CRISP2 remained associated with sperm after extraction with AM, a buffer with a composition favorable for the maintenance of the integrity of the AM. Finally, the results of detergent partitioning showing that CRISP2 was found in the aqueous phase after centrifugation indicated the hydrophilic nature of the protein.

To investigate the localization of CRISP2 after capacitation and the AR, aliquots of fresh, capacitated, and ionophore A23187-treated epididymal sperm were subjected to IIF using anti-CRISP2, as already described. The following two fluorescent patterns were observed in the three sperm populations: pattern A, showing bright staining localized in the acrosomal region, and pattern T, showing fluorescence localized to the sperm head tip (Fig. 2A). Quantification of fluorescent patterns in the different sperm populations indicated that, while most fresh sperm exhibited pattern A, the percentages of sperm with this pattern significantly decreased in capacitated and ionophore-treated sperm populations, with a concomitant increase in sperm showing pattern T. Similar percentages of unstained cells were detected in all the populations. In parallel to the IIF investigations, the occurrence of the AR in the different sperm populations was determined by staining of the acrosomal contents with Coomassie brilliant blue. The results indicated a correlation between the percentages of cells showing pattern T and the ARs in the three populations. To determine whether protein CRISP2 was proteolytically processed during sperm capacitation and the AR, the total protein contents from fresh, capacitated, and ionophore-treated epididymal sperm were subjected to SDS-PAGE and were examined by Western blot analysis. Results

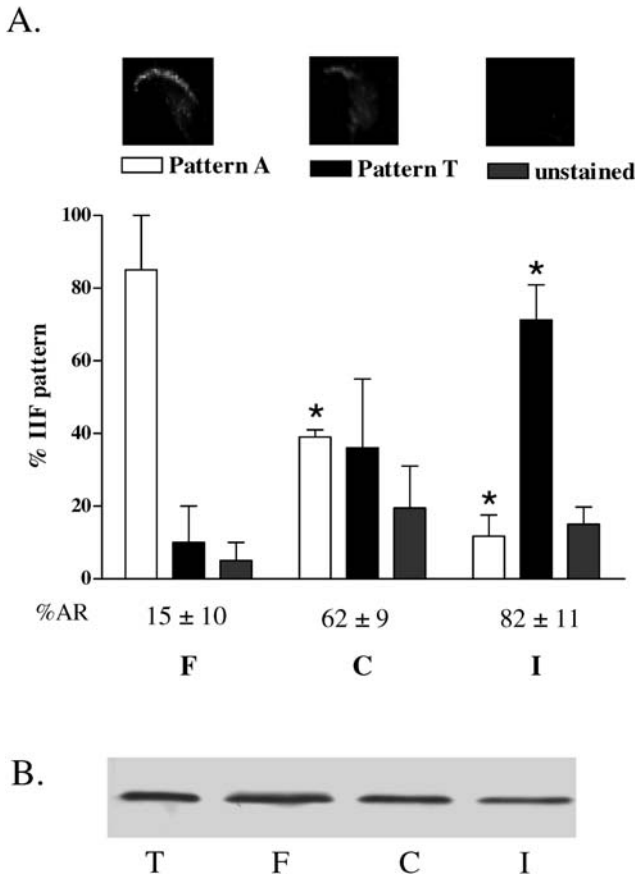

FIG. 2. Fate of mouse CRISP2 after capacitation and the AR. **A**) Percentages of fresh (F), capacitated (C), and ionophore-treated (I) epididymal sperm showing fluorescent labeling on the dorsal region (pattern A) or on the tip of the sperm head (pattern T) (n = 3; $*P < 0.05$ vs same pattern in F). IIF microphotographs representative of each pattern are shown above. The percentages of acrosome-reacted sperm (%AR) obtained by Coomassie brilliant blue staining are indicated at the bottom of the panel. Original magnification ×400. **B**) Mouse testicular cytosol (T) and protein extracts from fresh, capacitated, and ionophore-treated sperm separated by SDS-PAGE and subjected to Western blot analysis using anti-CRISP2.

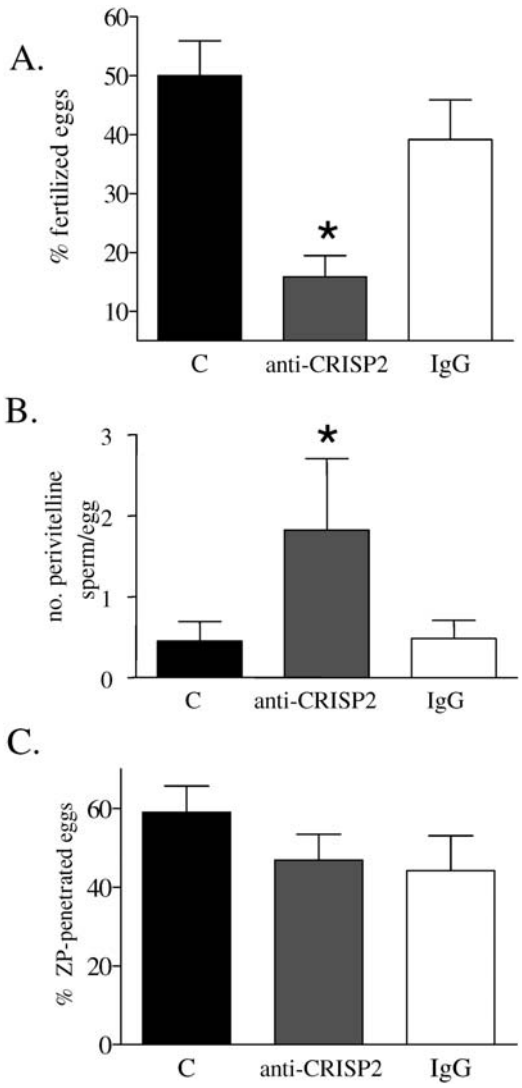

FIG. 3. Effect of anti-CRISP2 on mouse in vitro fertilization. ZP-intact eggs were inseminated with mouse-capacitated sperm preincubated in medium alone (C) or in medium containing anti-CRISP2 or normal rabbit IgG. After gamete coincubation, the following parameters were analyzed: **A**) Percentage of fertilized eggs. **B**) Number of perivitelline sperm per egg. **C**) Percentage of ZP-penetrated eggs (eggs with sperm in the perivitelline space or ooplasm) (n = 7; $*P < 0.05$).

showed a unique band of approximately 25 kDa in the three sperm populations (Fig. 2B).

Participation of CRISP2 in Fertilization

The retention of some CRISP2 immunofluorescence in acrosome-reacted sperm supported a potential involvement of this protein in sperm-egg interaction. To investigate this possibility, we evaluated the effect of anti-CRISP2 on the ability of mouse sperm to fertilize zona-intact eggs in vitro. Coincubation of gametes in the presence of the antibody resulted in a significant decrease in the percentage of fertilized eggs, which was not observed when anti-CRISP2 was replaced by normal rabbit IgG (Fig. 3A). This inhibition was accompanied by an increase in the number of perivitelline sperm per egg (Fig. 3B) and no differences in the percentage of ZP-penetrated eggs (eggs with sperm in the perivitelline space or ooplasm) (Fig. 3C), indicating an effect of the antibody at the level of sperm-oolema interaction.

To analyze whether the participation of CRISP2 at the sperm-oolema level was mediated by complementary sites in the egg, recombinant mouse protein CRISP2 was bacterially expressed as a fusion protein coupled to MBP (Fig. 4A). Analysis of the purified protein by silver staining and Western blot revealed the presence of a major band of the expected molecular weight for recCRISP2 (62 kDa) (Fig. 4B). Although a minor band of approximately twice the size of recCRISP2 was also detected, the absence of the band when SDS-PAGE was carried out under reducing conditions suggests that it could correspond to a dimer of the recombinant protein (data not shown). To study the presence of egg binding sites for CRISP2, ZP-intact mouse eggs were incubated with recCRISP2 and were then subjected to IIF using anti-MBP as primary antibody. Eggs exposed to MBP were used as controls. Results showed that eggs incubated with recCRISP2 exhibited fluorescent staining on their surface, which was not detected in MBP-incubated cells (Fig. 4Ca–e). IIF studies of zona-free mouse eggs exposed to recCRISP2 confirmed the presence of fluorescent staining at the surface level and revealed the existence of an unstained area of approximately one third of the cell surface (Fig. 4Cf).

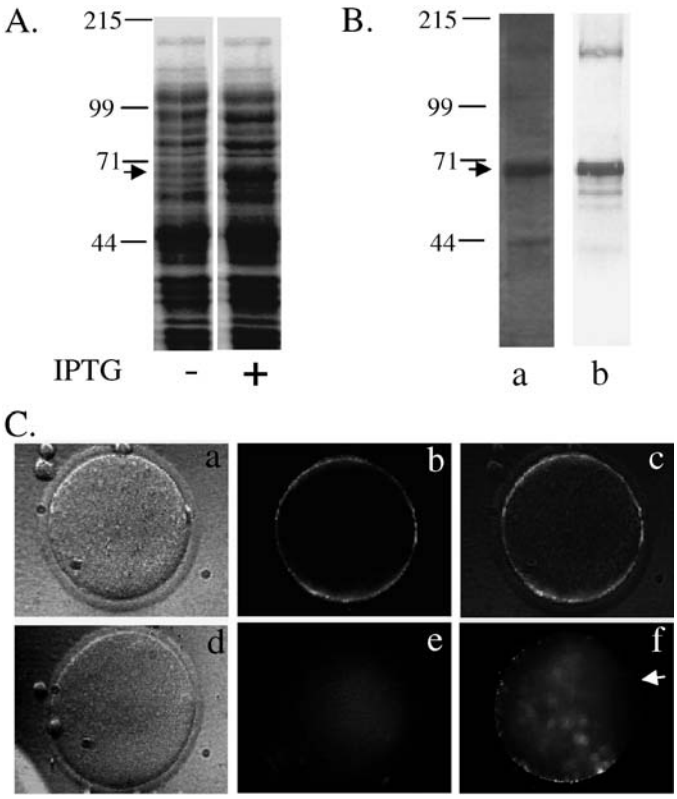

FIG. 4. Immunolocalization of binding sites for CRISP2 in mouse eggs. **A**) Expression of recCRISP2 analyzed by SDS-PAGE and Coomassie brilliant blue staining of bacterial protein extracts. The arrow indicates the presence of a protein of 62 kDa (expected molecular weight for recCRISP2) induced by IPTG. **B**) Purification of recCRISP2 analyzed by silver staining (a) and Western blot (b). **C**) Binding of recCRISP2 to eggs by IIF. Phase and fluorescent images of ZP-intact eggs incubated with recCRISP2 (a and b) and MBP (d and e) and then exposed to anti-MBP as primary antibody; (c) corresponds to superimposed-phase (a) and fluorescent (b) images. (f) Fluorescent image of a ZP-free egg incubated with recCRISP2. Original magnification ×200.

Considering the similar localization of rat CRISP1 and mouse CRISP2 complementary sites on the egg surface, as well as the high homology between these CRISPs, we next analyzed whether the two proteins were interacting with the same or different binding sites on the egg. For this purpose, ZP-free mouse eggs were incubated in medium containing a fixed concentration of recCRISP2 and increasing concentrations of rat CRISP1 and were then fixed and subjected to IIF using anti-MBP. The binding of rat CRISP1 to the membrane was confirmed by parallel IIF investigations of eggs incubated with this protein and the corresponding anti-CRISP1 antibody. Results showed that exposure of eggs to increasing concentrations of rat CRISP1 resulted in a gradual decrease in the level of IIF staining for recCRISP2 (Fig. 5), indicating that the proteins interact with common binding sites on the egg surface.

DISCUSSION

Although there is a general consensus about the specific expression of CRISP2 in testicular tissue and its presence in mature sperm, the functional role of this protein remains controversial. In the present study, we provide strong evidence supporting the involvement of CRISP2 in mouse sperm-egg fusion and the existence of binding sites for the protein on the egg surface.

Given the high interspecies conservation of CRISP2, we used an antibody produced against human recombinant CRISP2 to detect CRISP2 in rodent sperm. In this regard, the ability of anti-CRISP2 to recognize the mouse protein, as well as the absence of a crossreaction with the highly homologous rat epididymal protein CRISP1 in Western blot analysis, guaranteed the specificity of the results obtained with this antibody.

The need for permeabilization to detect immunofluorescent labeling in sperm suggests that mouse CRISP2 is mainly an intracellular component. This observation, together with the presence of the labeling in the acrosomal region of the sperm head, supports the intraacrosomal localization of mouse CRISP2, as previously reported for guinea pig [6], rat [31], and human [35] homologous proteins. Although weak labeling was observed in some sperm tails, no staining was detected in the midpiece region, a localization compatible with the reported presence of CRISP2 in the outer dense fibers of the rat sperm tail [31]. The use of different primary antibodies could be responsible for this divergence.

Our IIF results suggesting an intraacrosomal localization of mouse CRISP2 are in agreement with results obtained using different protein extraction procedures. The finding that a high ionic strength medium was ineffective in removing CRISP2 from sperm excludes a possible ionic interaction of this protein with the sperm surface. On the other hand, the results of TX-114 partitioning indicating the hydrophilic nature of CRISP2, together with the lack of hydrophobic transmembrane regions in the molecule, argue against a direct insertion of the protein into the membranes. Several sperm proteins present within the acrosome are linked to constitute a particulate structure, known as the AM, which is relevant for the structural integrity of the

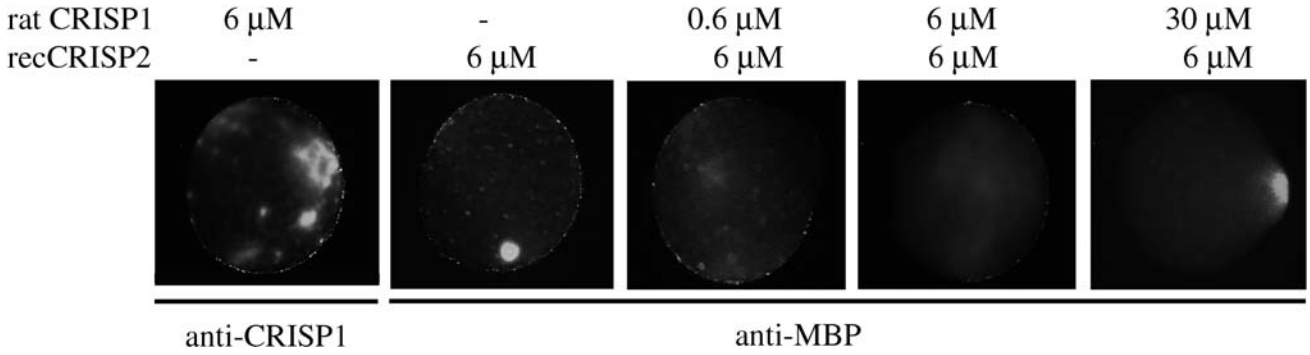

FIG. 5. Competitive binding of CRISP1 and CRISP2 to the egg surface. ZP-free mouse eggs incubated with 6 µM recCRISP2 and increasing concentrations of purified native rat CRISP1 were subjected to IIF using anti-CRISP1 or anti-MBP as primary antibodies. Original magnification ×200.

reacting acrosome and perhaps for the differential release of proteins during the AR [37, 44]. This matrix remains intact at acidic pH but dissolves by virtue of endogenous proteolytic activity at pH exceeding 7 [45, 46]. In agreement with this, our results showing that CRISP2 was completely released from mouse sperm at pH 11 but remained associated with the cells in acidic conditions suggest that the protein might be related to the AM. This was further supported by the observation that most of the protein remained in the pellet after extraction with AM buffer, a solution known to maintain the integrity of the AM. In this regard, CRISP3, another CRISP member highly homologous to CRISP2 (79%), was found to be a matrix protein within the granules of neutrophils [47]. Taken together, the results of our immunolocalization and protein extraction investigations support the intraacrosomal localization of mouse sperm CRISP2 and its specific association with the AM.

The fate of CRISP2 after capacitation was studied by correlating the localization of the protein in fresh, capacitated, and ionophore-treated epididymal sperm with the occurrence of the AR in each sperm population. The results of these investigations indicated that, while CRISP2 is mainly localized to the acrosomal region of fresh sperm, it remains associated with a specific region of the acrosome (referred to as the tip of the head) in acrosome-reacted sperm. It has been suggested that the mouse sperm tip corresponds to the anterior portion of the equatorial segment and might be involved in the initial contact between the sperm and egg membranes [48, 49].

Many sperm proteins have been shown to be processed during epididymal maturation or the activation of proteases that occurs during capacitation and the AR [50–52]. Moreover, it has been reported that a member of the CRISP family, protein CRISP3, is proteolytically cleaved during neutrophil granule exocytosis [11]. However, our results indicating that the electrophoretic mobility of mouse CRISP2 was identical in testicular tissue and in sperm subjected to capacitation or induction of the AR indicate that CRISP2 does not undergo posttranslational modifications during these functional events.

The persistence of CRISP2 in capacitated and acrosome-reacted sperm led us to explore the possible involvement of this protein in fertilization. Our results demonstrating that anti-CRISP2 significantly reduced the percentage of fertilized eggs, with a concomitant accumulation of sperm in the perivitelline space, strongly support the idea that CRISP2 participates in the event of sperm-egg fusion. In this regard, the finding that the antibody did not interfere with ZP penetration not only indicates the lack of participation of CRISP2 in this step of fertilization but also excludes a detrimental effect of the antibody on sperm motility or the AR, as only hyperactivated acrosome-reacted sperm are capable of penetrating the ZP.

Although it was traditionally believed that acrosomal proteins are mainly involved in ZP penetration, there are several studies showing that proteins inside the acrosome are important for gamete fusion. One example is the protease acrosin, which has been shown to bind to the equatorial segment of hamster sperm after the AR and to be relevant for the acquisition of the fusibility of this domain [53]. More recently, an acrosomal lysozyme-like protein called SLLP1 was also shown to play a role during mouse sperm-egg fusion [54]. A strong piece of evidence for the involvement of an acrosomal protein in gamete fusion comes from a recent report of the infertility of male knockout mice for a sperm acrosomal protein called Izumo, which is exposed only after the AR [55]. In these animals, sperm are able to bind to and penetrate the ZP but are incapable of fusing with the oolema and accumulate in the perivitelline space, as observed for anti-CRISP2-treated mouse sperm. According to results in human [35] and mouse (the present study), CRISP2 is an intraacrosomal protein involved in sperm-egg fusion.

Previous observations from our laboratory support the participation of other sperm CRISPs in the process of gamete fusion through their binding to specific sites on the egg surface (i.e., CRISP1 in rat [22] and mouse [28] and ARP [29] in human). In view of this, we decided to analyze whether the involvement of CRISP2 in mouse gamete fusion was also mediated by egg binding sites for the protein. IIF experiments indicated that recombinant CRISP2, produced and purified in our laboratory for this purpose, specifically bound to the oolema of zona-intact and zona-free mouse eggs. The fact that the ZP was completely unstained when exposed to the recombinant protein is in agreement with the lack of participation of CRISP2 in sperm-ZP interaction, inferred from our in vitro fertilization investigations.

The fluorescent pattern observed in zona-free eggs was coincident with that previously reported for rat CRISP1 in mouse eggs (i.e., labeling over the entire egg surface except for a negative area [28]). According to previous studies, this negative area would correspond to the region of the plasma membrane overlying the meiotic spindle [22], a region through which sperm rarely fuse with the egg [56, 57]. Because rat CRISP1 and mouse CRISP2 bind to the fusogenic area of mouse eggs and exhibit high homology in their amino acid sequences, we next investigated the possibility that these two proteins were interacting with a common binding site on the egg surface. Our in vitro competition investigations showed that exposure of the eggs to increasing amounts of rat CRISP1 gradually reduced the binding of recCRISP2 to the egg, suggesting that both CRISPs share complementary sites on the egg surface. These observations are in agreement with recent results from our group showing that the ability of rat CRISP1 to bind to the egg resides in a 12-amino acid region corresponding to a feature motif of the CRISP family [58]. The fact that 10 of 12 amino acids of this region are identical in mouse CRISP2 and rat CRISP1 provides a molecular basis to explain the interaction of the proteins with the same egg binding site. Although the results of our competition investigations might be due to the use of two proteins highly homologous in their egg binding domains, it is also possible that both CRISP1 and CRISP2 are required to mediate gamete fusion through their interaction with the egg. This last possibility is further supported by observations that both anti-CRISP1 [59] and anti-CRISP2 (the present study) significantly inhibit sperm-egg fusion, while being able to recognize only the corresponding protein. In addition, recent evidence indicates the functional cooperation of homologous molecules during fertilization. As suggested for CRISP1 and CRISP2 in sperm-egg fusion, fertilin and cyritestin, two members of the ADAM family, have been implicated in sperm-ZP interaction [60], while CD9 and CD81, two egg proteins belonging to the tetraspanin family, have been recently shown to play complementary roles in sperm-egg fusion [61]. Important information regarding the role of CRISP1 and CRISP2 in gamete fusion will be provided by functional studies in knockout mice for each of these proteins.

The results of the present study indicate that testicular protein CRISP2, as previously reported for epididymal protein CRISP1 [22], is relevant for sperm-egg fusion and is capable of interacting with complementary sites on the egg surface. The possible participation of these two CRISP proteins in sperm-egg fusion provides important information about the molecular mechanisms involved in this process and supports the idea of functional cooperation between homologous molecules as a mechanism to ensure the success of fertilization.

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