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Human Fertilization: Epididymal hCRISP1 mediates sperm-zona pellucida binding through its interaction with ZP3

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Abstract

Human epididymal CRISP1 (hCRISP1) associates with sperm during maturation and participates in gamete fusion through egg-complementary sites. Its homology with both rodent epididymal CRISP1 and CRISP4 reported to participate in the previous stage of sperm binding to the zona pellucida (ZP), led us to further investigate the functional role of hCRISP1 by studying its involvement in human sperm-ZP interaction. Human hemizona (HZ) were inseminated with human capacitated sperm in the presence of either anti-hCRISP1 polyclonal antibody to inhibit sperm hCRISP1, or bacterially-expressed hCRISP1 (rec-hCRISP1) to block putative hCRISP1 binding sites in the ZP. Results revealed that both anti-hCRISP1 and rec-hCRISP1 produced a significant inhibition in the number of sperm bound per HZ compared to the corresponding controls. The finding that neither anti-hCRISP1 nor rec-hCRISP1 affected capacitation-associated events (i.e. sperm motility, protein tyrosine phosphorylation, or acrosome reaction) supports a specific inhibition at the sperm-egg interaction level. Moreover, immunofluorescence experiments using human ZP-intact eggs revealed the presence of complementary sites for hCRISP1 in the ZP. To identify the ligand of hCRISP1 in the ZP, human recombinant proteins ZP2, ZP3 and ZP4 expressed in insect cells were co-incubated with hCRISP1 and protein-protein interaction was analyzed by ELISA. Results revealed that rec-hCRISP1 mainly interacted with ZP3 in a dose-dependent and saturable manner, supporting the specificity of this interaction. Altogether, these results indicate that hCRISP1 is a multifunctional protein involved not only in sperm-egg

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fusion but also in the previous stage of sperm-ZP binding through its specific interaction with human ZP3.

Key Words: fertilization/hCRISP1/oocyte/sperm/zona pellucida

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Introduction

Mammalian fertilization is a multistep process involving a well-orchestrated cascade of molecular events that culminates in the development of a new individual. In order to fertilize the egg, sperm must pass through the cumulus matrix, bind to and penetrate the zona pellucida (ZP) and, finally, fuse with the egg plasma membrane. Most of these cell-to-matrix and cell-to-cell interactions are mediated by specific molecules present in both gametes (Ikawa et al., 2010). One such molecule is epididymal sperm protein CRISP1, an androgen-regulated glycoprotein identified by our group in the rat epididymis (Cameo and Blaquier, 1976) and the first described member of the largely conserved CRISP (Cysteine-Rich Secretory Protein) family. Interestingly, CRISP proteins are highly enriched in the male reproductive tract of mammals in which four CRISPs (referred to as CRISP1, CRISP2, CRISP3, and CRISP4) have been identified (reviewed in: (Gibbs et al., 2008)).

Rat epididymal CRISP1 (32 kDa) associates with the sperm surface during epididymal maturation (Kohane et al., 1980a; Kohane et al., 1980b; Cameo et al., 1986), and it has been proposed to participate in both sperm-ZP interaction and sperm-egg fusion through its interaction with egg complementary sites (Rochwerger et al., 1992; Busso et al., 2007; Cohen et al., 2011). Subsequent studies showing that CRISP1 knockout sperm exhibited an impaired ability to penetrate both ZP-intact and ZP-free eggs, supported the proposed roles for CRISP1 during gamete interaction (Da Ros et al., 2008).

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In human, two different laboratories identified an epididymal secretory glycoprotein of low molecular weight (30 kDa) that binds to the sperm head during epididymal maturation (Hayashi et al., 1996; Kratzschmar et al., 1996). This protein was originally described as the human homologue of rodent CRISP1 and named hCRISP1 (Hayashi et al., 1996). These observations led our laboratory to explore whether hCRISP1 was involved in sperm-egg fusion as previously observed for rat CRISP1. Both the finding that an anti-hCRISP1 antibody significantly inhibited the ability of human sperm to penetrate ZP-free hamster eggs, and the existence of hCRISP1-binding sites in the surface of ZP-free human eggs supported the participation of hCRISP1 in gamete fusion (Cohen et al., 2001).

Few years later, two groups reported the existence of a new androgen-dependent epididymal protein called CRISP4 present in both mouse (Jalkanen et al., 2005) and rat (Nolan et al., 2006) sperm. The discovery of CRISP4 revealed that hCRISP1 exhibits a higher identity with rodent CRISP4 (60%) than with rodent CRISP1 (40%) (Nolan et al., 2006; Jalkanen et al., 2005). Interestingly, recent results revealed that CRISP4 null sperm exhibit a reduced ability to interact with the ZP (Turunen et al., 2012).

The homology of hCRISP1 with rodent epididymal CRISP1 and CRISP4 and the finding that both rodent proteins have been proposed to mediate sperm binding to the ZP (Busso et al., 2007; Turunen et al., 2012), opened the possibility for a role of hCRISP1 not only in gamete fusion (Cohen et al., 2001) but also in the previous step of

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human sperm-ZP interaction. In this regard, while it is known that the human ZP is composed of four glycoproteins i.e ZP1, ZP2, ZP3 and ZP4 (Lefievre et al., 2004) proposed to participate in sperm-ZP binding and/or acrosome reaction (Caballero-Campo et al., 2006; Chiu et al., 2008), less progress has been made in identifying the ZP receptors present in human sperm.

In view of this, the aim of the present study was to further investigate the biological function of hCRISP1 in fertilization by studying its participation in the sperm-ZP interaction stage. Evidence is presented supporting the involvement of hCRISP1 in human sperm binding to the ZP through its specific interaction with ZP3.

Materials and Methods

Ethical approval

The study protocol was approved by the Bioethics Committee of the Institute of Biology and Experimental Medicine (IBYME) from the National Research Council (CONICET). Human donors were provided with oral and written information about the study prior to giving an informed consent.

Expression of proteins

The expression of recombinant human CRISP1 (rec-hCRISP1) and rat CRISP1 (rec-CRISP1) coupled to maltose binding protein (MBP) was carried out in a prokaryotic system as previously described (Cohen et al., 2001; Ellerman et al., 2002). Recombinant

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MBP, expressed in the same system, was used as control. Recombinant human ZP proteins (ZP2, ZP3, and ZP4) were expressed in the *Spodoptera frugiperda* Sf9 insect ovary cell line using the baculovirus expression system previously described (Carino et al., 2002).

Human sperm capacitation

Semen samples from adult (21-35 years old) healthy normospermic donors (WHO, 2010) were obtained by masturbation into a sterile plastic container after 48 h of sexual abstinence. After complete liquefaction, the semen was diluted with six volumes of Biggers, Whitten, Whittingham (Biggers et al., 1971) (BWW) medium, centrifuged at 300 X g for 10 min. Sperm samples were then allowed to swim up in 1 ml of BWW containing 3.5% human serum albumin (BWW-HSA, Sigma-Aldrich, St Louis, MO) for 1 h at 37˚C in an atmosphere of 5% CO_2, and highly motile selected sperm were diluted in the same medium (adjusting the final sperm concentration to 5-10 x10^6 cells/ml) and incubated for 18 h at 37˚C in an atmosphere of 5% CO_2.

Hemizona Assay (HZA)

Human oocytes were obtained by follicular aspiration from women undergoing ovulation stimulation for the purpose of assisted fertilization. Either non-inseminated immature oocytes incubated in HTF medium (Irvine Scientific, Santa Ana, CA) for *in vitro* maturation or oocytes that failed to be fertilized by either conventional IVF or ICSI, were stored in a solution containing 0.1 M Tris, 1.5 M $(NH_4)_2SO_4$ and 0.5%

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dextran (pH 7), at 4°C until use. This form of storage has been demonstrated to retain the biological characteristics of the ZP (Yanagimachi et al, 1979, Fayrer-Hosken and Brackett, 1987). On the day of the experiment, oocytes were thoroughly washed in BWW-HSA, mounted on a phase contrast inverted microscope (Nikon, Diaphot, Tokyo, Japan), and bisected into equal halves (hemizona, HZ) by using a microscalpel attached to micromanipulators (Narishigue, Tokyo, Japan). In all cases, one HZ was exposed to the treatment while the matching HZ was incubated under control conditions. Each HZ was placed in a 100 µl drop of BWW-HSA and co-incubated with 3 x 10^4 capacitated sperm for 4 h at 37°C in an atmosphere of 5% CO_2 (Burkman et al., 1988). To evaluate the effect of the rabbit polyclonal antibody against recombinant hCRISP1 (anti-hCRISP1) (Cohen et al., 2001) on sperm-ZP binding, HZ were added to a drop containing capacitated sperm that had been pre-incubated for 30 min in medium containing anti-hCRISP1 or normal rabbit immunoglobulin G (IgG) (Sigma-Aldrich, St. Louis, MO). For those experiments aimed to analyze the effect of rec-hCRISP1, HZ were pre-incubated for 30 min in a drop of medium alone or medium containing either rec-hCRISP1 or MBP prior to their insemination with capacitated sperm. At the end of all gamete co-incubations, HZ were removed and washed by repeated vigorous pipetting to detach loosely associated sperm, and the number of sperm tightly bound to the outer surface of the HZ was determined under the microscope. For each treatment (anti-hCRISP1 or hCRISP1), at least three independent experiments were performed on separate days using sperm from different donors.

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Computer-Assisted Semen Analysis (CASA)

Aliquots of 5 µl of capacitated sperm suspensions incubated for an additional 4 h in medium alone or medium containing rec-hCRISP1 were placed in a Makler chamber (Sefi-Medical Instruments, Rehovot, Israel), and motility parameters were measured in a computerized sperm analyzer (Hamilton Thorn IVOS/09279/V10.8s; Hamilton Thorn, Danvers, MA). For each sample, the mean value of more than 200 cells (from at least eight different fields) was calculated. The following parameters were measured: motility (percentage), progressive motility (percentage), average path velocity (VAP, µm/sec), straight-line velocity (VSL, µm/sec), track speed (VCL, µm/sec), amplitude of lateral head displacement (ALH, µm), beat-cross frequency (BCF, Hz), straightness (STR = VSL/VAP), and linearity (LIN = VSL/VCL). In addition, sperm were distributed into four categories according to their velocities (Rapid, VAP >25µm/s; Medium, 25µm/s >VAP>5µm/s; Slow, VAP <5µm/s; Static, sperm not moving).

Sperm extracts and Western blotting analysis

Fresh or capacitated human sperm were incubated for 4 h in medium either alone or containing 6 µM of rec-hCRISP1. At the end of the incubation, sperm were washed with PBS, incubated with Laemmli sample buffer (Laemmli, 1970) for 5 min, and then boiled and centrifuged at 5000 X g for 5 min. The supernatants were recovered and boiled again in the presence of 70 mM 2-β-mercaptoethanol. Sperm protein extracts (corresponding to 1 x 10^6 cells) were then separated by SDS–PAGE (Laemmli, 1970) and electrotransferred onto nitrocellulose membranes (Towbin et al., 1979). After

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blocking with PBS containing 2% skim milk and 0.1% Tween 20, the membranes were probed with the anti-phosphotyrosine monoclonal antibody (1:5000; clone 4G10; Upstate, Lake Placid, NY) followed by peroxidase-conjugated secondary antibody (1:4000; Vector, Burlingame, CA). Monoclonal antibodies against β tubulin (1:50000; clone D66, Sigma-Aldrich St Louis, MO) were used to assess protein loading. In all cases, the immunoreactive proteins were detected using an ECL Western Blotting kit (Amersham Life Science, Oakville, Canada).

Evaluation of acrosomal status

Human capacitated sperm were incubated for 4 h in medium either alone or containing 6 μM of rec-hCRISP1. At the end of the incubation, sperm were exposed to either progesterone (25 μM) (Sigma-Aldrich St Louis, MO) or DMSO (J.T. Baker, Phillipsburg, USA) for additional 30 min, and the acrosomal status assessed by staining with FITC-labeled *Pisum sativum* agglutinin (PSA; Sigma-Aldrich St Louis, MO) as previously described (Cohen et al., 2001). Sperm were scored as “acrosome intact” when a bright staining was observed in the acrosome or as “acrosome reacted” when either the fluorescent staining was restricted to the equatorial segment or no labeling was observed.

Indirect immunofluorescence (IIF) of human oocytes

Zona-intact oocytes obtained as described above were incubated for 30 min at 37°C in medium alone or in medium containing 6 μM rec-hCRISP1 or rat rec-CRISP1. At the

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end of the incubation, the oocytes were washed with medium, fixed for 45 min in 2% (w/v) paraformaldehyde in PBS at room temperature, washed again, incubated for 30 min at 37°C in 5% (v/v) normal goat serum (NGS) in PBS containing 4 mg/ml BSA (PBS-BSA4, Sigma-Aldrich St Louis, MO) and exposed to anti-MBP (1:100; New England BioLabs, Inc., Beverly, MA) for 1 h at 37°C. After washing in PBS-BSA4, eggs were incubated for 30 min at 37°C in FITC-conjugated goat-anti-rabbit IgG (1:50 in PBS-BSA4), washed, mounted in 90% glycerol in PBS, and finally examined with a Nikon Optiphot microscope (Nikon, Tokyo, Japan) equipped with epifluorescence optics.

Enzyme-Linked Immunosorbent assay (ELISA)

Purified recombinant ZP proteins (ZP2, ZP3, ZP4) diluted in PBS were coated onto a 96-well microtiter plate (Nunc A/S, Roskilde, Denmark) (0.05 µg/well), and incubated overnight at 4ºC. In those cases in which a combination of two ZP proteins was tested, half the amount of each ZP was coated in the well. After blocking with PBS containing 2% BSA for 90 min at 37ºC, 50 µl of PBS containing 0.1% BSA (PBS-BSA 0.1%) or containing either rec-hCRISP1 (100-900 ng/well) or equimolar amounts of MBP were placed in duplicate wells and incubated for 90 min at 37ºC. Wells were then washed three times with PBS and fixed with 0.1% p-formaldehyde for 30 min at room temperature to avoid loss of captured proteins. After washing, wells were incubated with anti-MBP (1:2000 in PBS-BSA 0.1%) for 90 min at 37 °C, followed by biotin-conjugated anti-rabbit secondary antibody (1:500 in PBS-BSA 0.1%) for 1 h at 37ºC.

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Wells were then washed, and subsequently exposed to ExtrAvidin-alkaline phosphatase (Sigma-Aldrich St Louis, MO; 1:1000 in PBS-BSA 0.1%) for 30 min at room temperature. After rinsing, color reaction was allowed to develop by addition of p-nitro-phenyl-phosphate (1 mg/ml in 10% v/v diethanolamine, 0.4 mM $MgCl_2$, pH 9.8) to each well. Absorbance at 405 nm was determined with a microplate reader (Cambridge Technology, Inc., Watertown, MA). Wells without ZP proteins and subjected to the same procedures were used as background wells.

Statistical analysis

Paired Student's t-test was used to compare the values of sperm–ZP binding among different treatment groups. Sperm motility parameters were analyzed by multiple unpaired Student's t-test. The percentages of acrosome-reacted sperm were analyzed by the X^2 test. ELISA results were analyzed by one-way ANOVA and Tukey´s multiple comparison test. All calculations were performed using the Prism 4.0 software (GraphPad Software, La Jolla, CA, USA). Results were expressed as mean values ± SEM for each series of experiments and were considered to be significantly different at $P<0.05$. 'n' refers to the number of independent experiments.

Results

The potential involvement of hCRISP1 in sperm-ZP interaction was evaluated by the HZA, which involves the use of matching halves of human ZP from non-living oocytes, providing an internal control on human ZP variability. As a first approach, the HZ were

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co-incubated with human capacitated sperm in the presence of a specific antibody against hCRISP1 (anti-hCRISP1) at a concentration known to inhibit the ability of human sperm to penetrate ZP-free hamster eggs (1:50) (Cohen et al., 2001). The matching HZ co-incubated with sperm in either medium alone or medium containing normal rabbit IgG were used as control. Results showed that the presence of anti-hCRISP1 during gamete co-incubation produced a significant ($p<0.005$) inhibition in the number of sperm bound per HZ compared to controls (Figure 1A). No further inhibition was observed at a higher antibody concentration (1:10). The inhibitory effect was not due to a deleterious effect of anti-hCRISP1 on sperm as judged by the fact that, as previously reported (Cohen et al., 2001), the antibody neither induced sperm agglutination nor affected sperm viability, motility or acrosome reaction (data not shown).

As a second approach to the same question, we analyzed the effect of purified rec-hCRISP1 on gamete interaction with the premise that if complementary sites for hCRISP1 exist in the human ZP, exposure of the HZ to rec-hCRISP1 should competitively inhibit gamete interaction. In this set of experiments, gametes were co-incubated in medium alone or in medium containing either rec-hCRISP1, at a concentration known to bind to the complementary sites in the human oolema (6 μM) (Cohen et al., 2001), or an equimolar concentration of MBP as control. As shown in Figure 1B, the presence of rec-hCRISP1 during gamete interaction significantly ($p <0.001$) reduced the number of sperm bound per HZ compared to controls.

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To exclude a possible deleterious effect of rec-hCRISP1 on sperm during gamete interaction, capacitated sperm were incubated with rec-hCRISP1 for 4 h and different sperm functional parameters analyzed. Objective evaluation of sperm motility by CASA revealed that sperm exposed to rec-hCRISP1 presented no differences in any of the sperm motion parameters analyzed compared to controls (Table I). Exposure of sperm to rec-hCRISP1 had no effect on sperm protein tyrosine phosphorylation, a key event of capacitation (Figure 2A). As it has been reported that acrosome-reacted sperm do not bind to the human ZP (Liu et al., 2006), the effect of rec-hCRISP1 on sperm acrosome reaction was also analyzed. Results revealed that exposure of sperm to the protein did not affect the occurrence of either the spontaneous or progesterone-induced acrosome reaction (Figure 2B), excluding a decreased sperm ZP-binding ability due to defects in sperm capacitation-associated events.

According to these results, the inhibition in sperm-ZP binding produced by rec-hCRISP1 was indicative of the existence of hCRISP1-binding sites in the human ZP. This possibility was explored by IIF experiments in which human ZP-intact eggs were incubated with medium either alone or containing rec-hCRISP1, and then exposed to anti-MBP as primary antibody. As a control of protein-binding specificity, ZP-intact eggs were exposed to rat rec-CRISP1, another MBP-tagged CRISP protein expressed in the same prokaryotic system than rec-hCRISP1. Whereas control eggs exposed to

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medium alone or rat rec-CRISP1 were negative, those incubated with hCRISP1 showed a clear fluorescent labeling in both the ZP and the oolema (Figure 3).

With the aim of investigating the molecular mechanisms underlying the involvement of hCRISP1 in human sperm-ZP binding, a series of experiments were performed using recombinant ZP2, ZP3 and ZP4 proteins expressed in Sf9 insect cells and previously reported to be biologically active (Caballero-Campo et al., 2006). For these studies, each of the purified ZP proteins was immobilized in individual wells and co-incubated with either rec-hCRISP1 or MBP, and protein-protein interactions were analyzed by ELISA using anti-MBP as primary antibody. The observation that anti-MBP recognized both rec-hCRISP1 and MBP but did not cross-react with any of the recombinant ZP proteins (Figure 4A), supports the use of this antibody for the purposes of this study. Results revealed absorbance values significantly ($p < 0.001$) higher when rec-hCRISP1 was exposed to ZP3 than to ZP2 or ZP4, and an almost undetectable reaction when MBP was added to any of the ZP proteins analyzed (Figure 4B). The use of ZP3 combined with either ZP2 or ZP4 did not increase the binding of hCRISP1 to ZP3 alone (data not shown).

To further characterize the interaction between hCRISP1 and ZP3, recombinant ZP3 was immobilized in the well and then exposed to different concentrations of rec-hCRISP1. Results shown in Figure 4C indicated that the signal intensity increases as a function of the concentration of rec-hCRISP1 added and reached a maximum at

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600ng/well, revealing that hCRISP1 interacts with ZP3 in a dose-dependent and saturable manner. In contrast, MBP did not show reactivity with ZP3 even at the highest concentration tested.

Discussion

Previous studies from our laboratory support the involvement of hCRISP1 in gamete fusion through complementary sites localized on the surface of the human oocyte (Cohen et al., 2001). Based on the homology of hCRISP1 with rodent epididymal CRISP1 and CRISP4, both reported to be involved in sperm-binding to the ZP (Busso et al, 2007, Turunen et al, 2012), in the present work we have further investigated the biological function of hCRISP1 by studying its participation in sperm binding to the human ZP.

The evaluation of sperm binding to the ZP was carried out using the HZA, a unique internally controlled bioassay that evaluates tight binding of human spermatozoa to matching halves of a human ZP avoiding the inherent differences among human oocytes. Moreover, evidence indicates that the HZA exhibits high species-specificity and high predictive value for human IVF outcomes (Oehninger et al., 1993; Oehninger et al., 2013). The HZA studies were carried out in the presence of either anti-hCRISP1 or purified rec-hCRISP1 during gamete co-incubation with the idea of blocking hCRISP1 in sperm or putative hCRISP1-binding sites in the ZP, respectively. In both cases, results revealed a significant reduction in the number of sperm bound to the ZP

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not observed when a control antibody or protein was used. Although the existence of a steric hindrance by the antibody cannot be ruled out, the observation that sperm binding to the ZP was also affected by the presence of the recombinant protein during gamete co-incubation does not favor this possibility. In addition, as previously reported (Cohen et al., 2001), anti-hCRISP1 did not produce sperm agglutination or detrimental effects on sperm viability, motility or acrosome reaction. Similarly, rec-hCRISP1 did not affect sperm motility parameters evaluated by CASA nor capacitation-associated events such protein tyrosine phosphorylation and spontaneous or induced acrosome reaction, excluding the possibility that the presence of the protein during gamete co-incubation was detrimental to sperm or interfered with sperm capacitation. This is an important observation considering that both CRISP1 and CRISP4 null sperm present altered capacitation-associated events. While CRISP1 KO sperm show lower levels of protein tyrosine phosphorylation (Da Ros et al., 2008), sperm from CRISP4 KO mice exhibit an impaired ability to undergo progesterone-induced acrosome reaction (Gibbs et al., 2011, Turunen et al., 2012). Moreover, considering reports indicating that acrosome-reacted sperm do not bind to the human ZP (Liu et al., 2006), our observations also exclude the possibility that sperm-ZP binding inhibition was due to an increased level of acrosome reaction induced by rec-hCRISP1. Together, the results obtained using the HZA approach support the specific involvement of hCRISP1 in sperm-ZP binding through the interaction of the protein with complementary sites in the human ZP. This possibility was further supported by IIF localization studies showing a clear fluorescent labelling in the ZP of those eggs incubated with rec-hCRISP1 in contrast to the negative

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reaction observed in those eggs exposed to rat rec-CRISP1, another CRISP protein expressed as a MBP-fused molecule in the same prokaryotic system than rec-hCRISP1. Furthermore, the hCRISP1-treated eggs also exhibited staining at the plasma membrane level consistent with our previous observations (Cohen et al., 2001).

In order to gain insights into the molecular mechanism involved in the participation of hCRISP1 in sperm-ZP interaction, we investigated the putative hCRISP1 ligand in the egg glycoprotein matrix. Considering the limited availability of human ZP, recombinant human ZP2, ZP3 and ZP4 expressed in Sf9 insect cells were used for these studies. It is important to note that these recombinant proteins are biologically active as judged by their ability to both bind to human capacitated sperm (Chirinos et al., 2011) and promote the human acrosome reaction (Caballero-Campo et al., 2006). Interestingly, our studies revealed that rec-hCRISP1 exhibited a significantly higher ability to bind to ZP3 than to ZP2 or ZP4 which was not enhanced by the combined use of ZP3 with the other two ZP proteins. The possibility that the binding of hCRISP1 to ZP3 could be mediated through the MBP portion of the molecule was excluded as MBP showed no ability to interact with any of the recombinant ZP proteins. These results and the fact that hCRISP1 bound to ZP3 in a dose-dependent and saturable manner, support the specificity of the interaction observed. In the last few years, there have been reports showing binding of human sperm not only to ZP3 but also to ZP4 or ZP2 (Chiu et al., 2008; Chirinos et al., 2011; Baibakov et al., 2012). In addition, different models for explaining the interaction of intact or acrosome-reacted sperm with the different ZP

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molecules have been proposed (Clark, 2011; Avella et al., 2013). Our results showing that hCRISP1 binds mainly to ZP3 is consistent with the surface acrosomal localization of this epididymal protein in human (unpublished observations) and non human primate (Ellerman et al, 2010) sperm and support the idea that other sperm proteins may be involved in sperm binding to ZP2 or ZP4.

Evidence in several species including human suggests that specific interactions between sperm and ZP are mainly carbohydrate-mediated events (Pang et al., 2011; Clark, 2013). In this regard, the recombinant ZP proteins used in our studies are expressed in insect cells and thus, are glycosylated. Moreover, lectin analysis revealed that these recombinant ZP proteins underwent a type and degree of glycosylation that differs only slightly from their native counterparts (Chirinos et al., 2011). In view of this, the recombinant ZP proteins used in this study represent suitable tools to investigate the mechanisms underlying human sperm-ZP binding.

The finding that the bacterially-expressed hCRISP1 has the ability to bind to both native ZP (by IIF) and isolated ZP3 (by ELISA) suggests that the carbohydrate portion of the molecule would not be essential for its interaction with ZP. This conclusion is in agreement with our observations in rodents showing that deglycosylated native CRISP1 is capable of binding to the ZP (Busso et al., 2007). In this regard, the observation that the polypetidic portion of hCRISP1 has the ability to bind to ZP3 opens the possibility

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of performing further structure-function studies using recombinant fragments and synthetic peptides to identify the ZP binding site within hCRISP1.

Despite the numerous reports aimed towards dissecting the molecular mechanism underlying mammalian sperm-ZP interaction, there is still considerable controversy about the sperm receptors involved in this stage of fertilization (Avella et al., 2013). Different sperm components (i.e. galactosyltransferase (Shur and Hall, 1982), zonadhesin (Lea et al., 2001), P26h (Montfort et al., 2002), SED1 (Ensslin and Shur, 2003), sp56 (Cohen and Wassarman, 2001; Kim et al., 2001), CRISP1 (Busso et al., 2007)) have been proposed to mediate sperm-ZP binding in rodents, suggesting the existence of protein redundancy as a mechanism to ensure the success of fertilization. Consistent with this, several human sperm proteins have been proposed to participate in human sperm binding to the ZP (Sullivan et al., 2006; Naz and Dhandapani, 2010; Chiu et al., 2007) or, as shown in this study, in the specific interaction between human sperm and ZP3 (Naz et al., 1991; Petit et al., 2013). These observations, together with recent findings proposing that chaperones coordinate the formation of multimeric ZP recognition complexes on human sperm surface (Redgrove et al., 2011; Redgrove et al., 2013) led us to speculate that hCRISP1 could be part of a large protein complex involved in human sperm binding to the ZP.

The finding that hCRISP1 is involved not only in gamete fusion but also in sperm binding to the ZP supports the idea that this protein may represent the human molecule

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equivalent to both rodent CRISP1, involved in gamete fusion (Cohen et al., 2001) and sperm-ZP binding (Busso et al., 2007), and rodent CRISP4 reported to be involved in sperm-ZP interaction (Turunen et al., 2012). This hypothesis is in line with the fact that while two epididymal CRISP proteins are present in rodent sperm (i.e CRISP1 and CRISP4), hCRISP1 is the only epididymal CRISP described in the human male gamete.

In current assisted reproductive technology used to circumvent human infertility, complete failure of fertilization occurs in 10-15% of treatments when standard *in vitro* fertilization is used (Liu and Baker, 1994). These fertilization failures are mostly attributed to defects in the ZP binding and ZP penetration stages (Liu and Baker, 2000). Therefore, the identification of hCRISP1 as a molecule involved in human sperm-ZP interaction may contribute to the development of new methods of diagnosis and treatment of cases of idiopathic male infertility. In this regard, Sullivan and collaborators (2006) have shown that levels of P34H, a sperm epididymal protein involved in sperm-ZP binding, is predictive of *in vitro* fertilization outcome.

Immunization of monkeys with rec-hCRISP1 revealed the presence of specific antibodies against hCRISP1 both in seminal plasma and on ejaculated sperm from immunized animals, indicating the entry of the antibodies to the male reproductive tract and their ability to bind to sperm (Ellerman et al., 2010). These results together with the finding that anti-hCRISP1 antibodies are capable of inhibiting the ability of sperm to bind to the ZP (this study) and to fuse with the oolema (Cohen et al., 2001) support

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both the involvement of anti-hCRISP1 antibodies in human immunoinfertility and hCRISP1 as a likely candidate for immunocontraception. In this regard, a contraceptive method that does not affect sperm production and/or libido but specifically interferes with the sperm fertilizing ability represents an attractive and promising approach. Moreover, the participation of hCRISP1 in two different stages of the fertilization process (i.e. sperm-ZP binding and gamete fusion) increases the chances of interference and the potential effectiveness of the method.

In summary, the results of the present work provide evidence supporting that hCRISP1 is a multifunctional protein involved not only in gamete fusion but also in the previous stage of sperm-ZP binding through its specific interaction with ZP3. We believe this information will contribute to a better understanding of the molecular mechanisms underlying human fertilization as well as to the development of new methods for both diagnosis/treatment of infertility and fertility regulation in men.

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Authors´roles: P.S.C conceived the study. J.A.M, M.W.M, M.C, D.B, M.A.B and F.G.R designed and performed the experiments and data analysis. F.G.R, J.A.B, M.C and F.L provided the human samples and expertise. M.W.M helped in drafting the article. J.A.M and P.S.C wrote the article. All authors contributed to the final version of the manuscript.

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Figure legends

Figure 1: Effect of anti-hCRISP1 and rec-hCRISP1 on human sperm-ZP interaction. (A) Human HZ were inseminated with capacitated sperm pre-incubated in medium either alone (empty triangles) or containing normal rabbit IgG (1:50) (filled triangles) as controls, or in medium containing anti-hCRISP1 (1:50) (filled circles). (B) Human HZ were pre-incubated in medium either alone (empty triangles) or containing 6 μM of maltose-binding protein (MBP) (filled triangles) as controls, or in medium containing 6 μM rec-hCRISP1 (filled circles), and then were inseminated with capacitated sperm. In all cases, at the end of gamete incubation, HZ were washed by repeated vigorous pipetting and the number of bound sperm was counted. In both A and B, at least three independent experiments were performed using sperm from different donors A: $p<0.005$ vs. control; B: $p <0.001$ vs control.

Figure 2: Effect of rec-hCRISP1 on sperm protein tyrosine phosphorylation and acrosome reaction. (A) Total protein extracts from equal amounts of fresh (F) or capacitated (C) sperm incubated for 4 h in the absence (-) or presence (+) of 6 μM of rec-hCRISP1 were analyzed by Western blotting using an anti-phosphotyrosine antibody (*upper panel*). β-tubulin was used as loading control (*bottom panel*). The blot shown is representative of 3 independent experiments. (B) Capacitated sperm were incubated for 4 h in medium either alone (-) or containing 6 μM of rec-hCRISP1 (+), and the percentage of spontaneous and progesterone (P4)-induced acrosome reaction

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were evaluated by staining sperm with FITC-labelled *Pisum sativum* agglutinin (PSA). Results represent the mean ± SEM of 3 independent experiments. No significant differences among groups were observed.

* $p < 0.05$ vs spontaneous acrosome-reaction.

Figure 3: Binding of rec-hCRISP1 to ZP-intact human eggs. Phase and fluorescent images of ZP-intact human eggs incubated in medium containing 6 µM of either rat rec-CRISP1 (A, B) or rec-hCRISP1 (C, D) and subjected to indirect immunofluorescence (IIF) using anti-MBP as primary antibody. At least 10 oocytes per treatment were used. Note the fluorescent labeling in the ZP (arrow) and oolema (arrowhead) in D, and the absence of fluorescent labelling in B. Eggs incubated in medium alone exhibited the same pattern as those shown in B. Magnification X200. Scale Bar: 15µM

Figure 4: Binding of rec-hCRISP1 to human recombinant ZP proteins analyzed by ELISA. (A) Wells coated with rec-hCRISP1, MBP, ZP2, ZP3 or ZP4 (50 ng/well) were exposed to anti-MBP as first antibody. (B) Human recombinant proteins ZP2, ZP3 and ZP4 were immobilized on a microtiter plate (50 ng/well), co-incubated with either rec-hCRISP1 (300 ng /well), equimolar amounts of MBP or medium alone, and then exposed to anti-MBP. (C) Human recombinant ZP3 was immobilized on a microtiter plate (50 ng/well), co-incubated with increasing concentrations of rec-hCRISP1 or MBP at the highest concentration as control, and then exposed to anti-MBP. Results represent the mean value ± SEM of at least 3 independent experiments. *$p < 0.001$.

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Table I: Effect of rec-hCRISP1 on sperm motility analyzed by CASA

Parameters analyzed	medium	rec-hCRISP1
Motility (%)	86,3 ± 1,1	83,3 ± 3,9
Progressive motility (%)	58,0 ± 4,2	57,7 ± 5,8
VAP (µm/sec)	78,3 ± 6,7	82,5 ± 7,9
VSL (µm/sec)	64,6 ± 6,3	68,1 ± 7,7
VCL (µm/sec)	134,3 ± 12,8	146,6 ± 13,3
ALH (µm)	5,6 ± 0,4	6,4 ± 0,4
BCF (Hz)	20,7 ± 1,2	21,8 ± 1,8
STR	81,5 ± 1,5	81,7 ± 1,8
LIN	49,8 ± 1,9	48,0 ± 1,5
rapid (%)	84,5 ± 1,2	80,7 ± 3,7
medium (%)	1,7 ± 0,3	2,7 ± 0,3
slow (%)	4,0 ± 0,4	5,0 ± 1.0
static (%)	10,3 ± 1,6	12,0 ± 4,0

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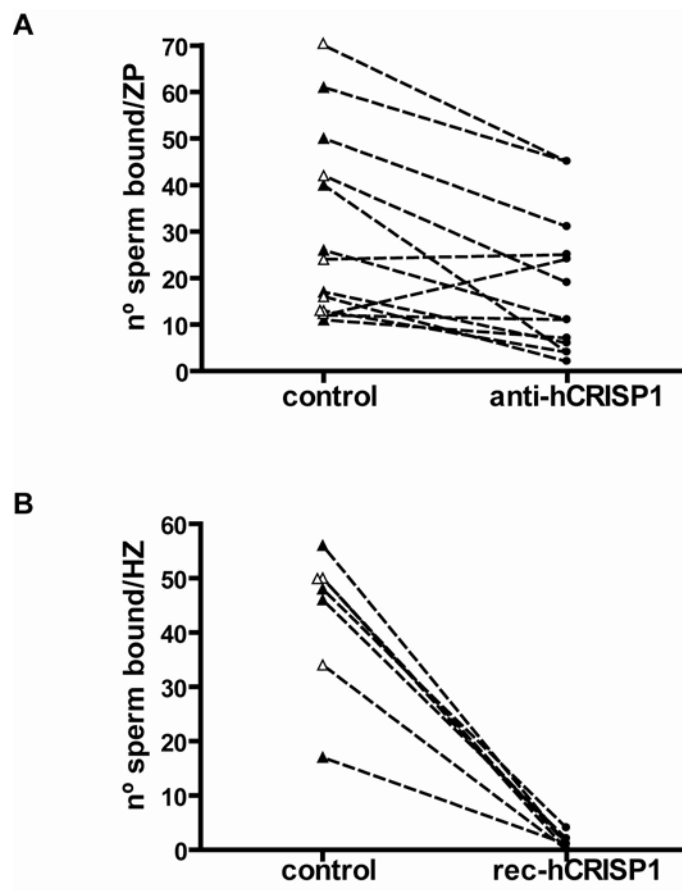

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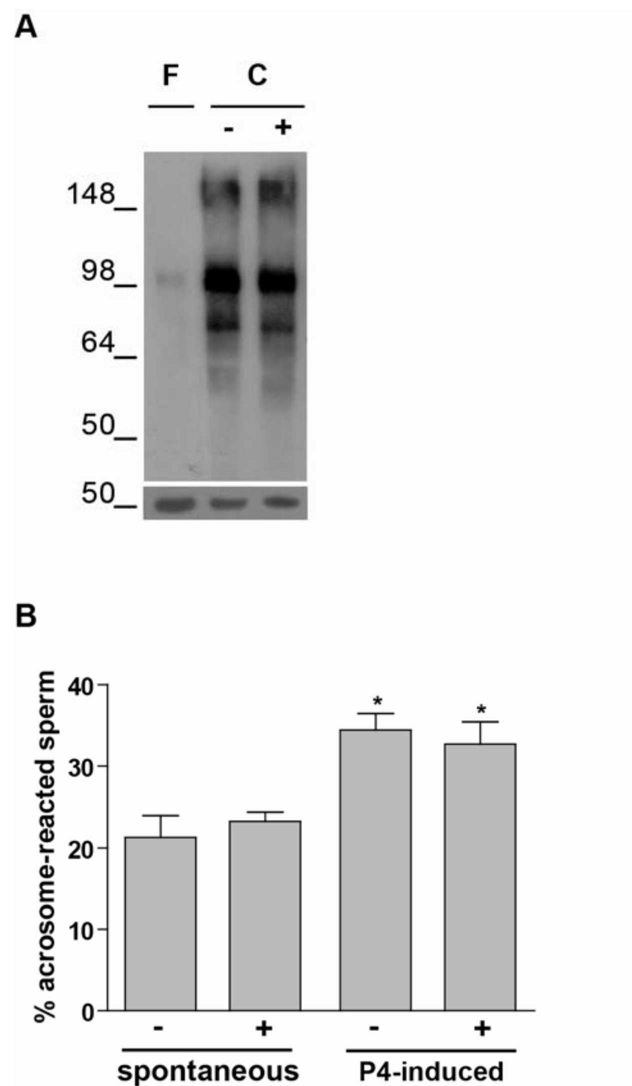

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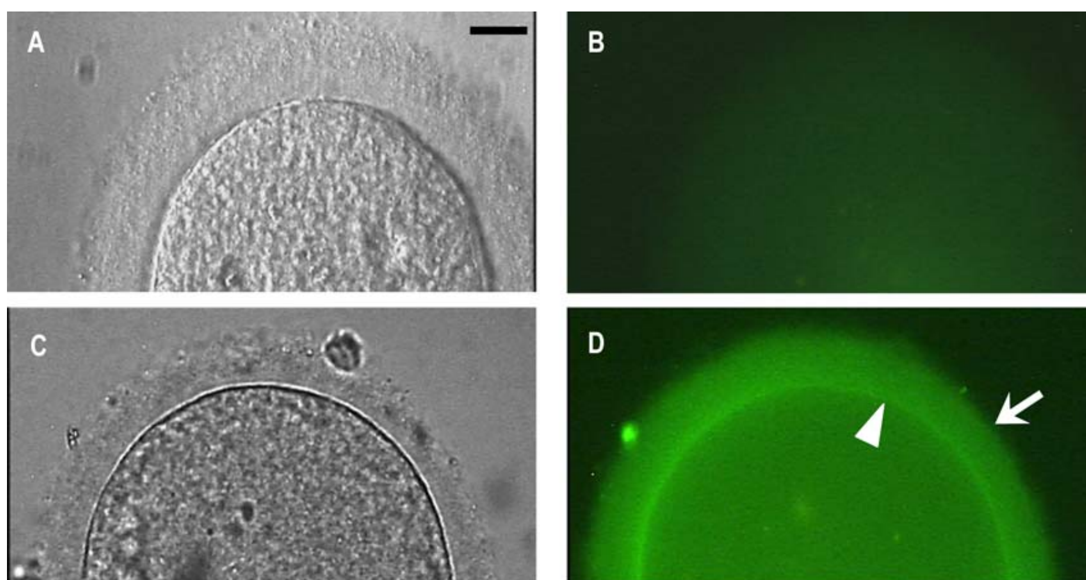

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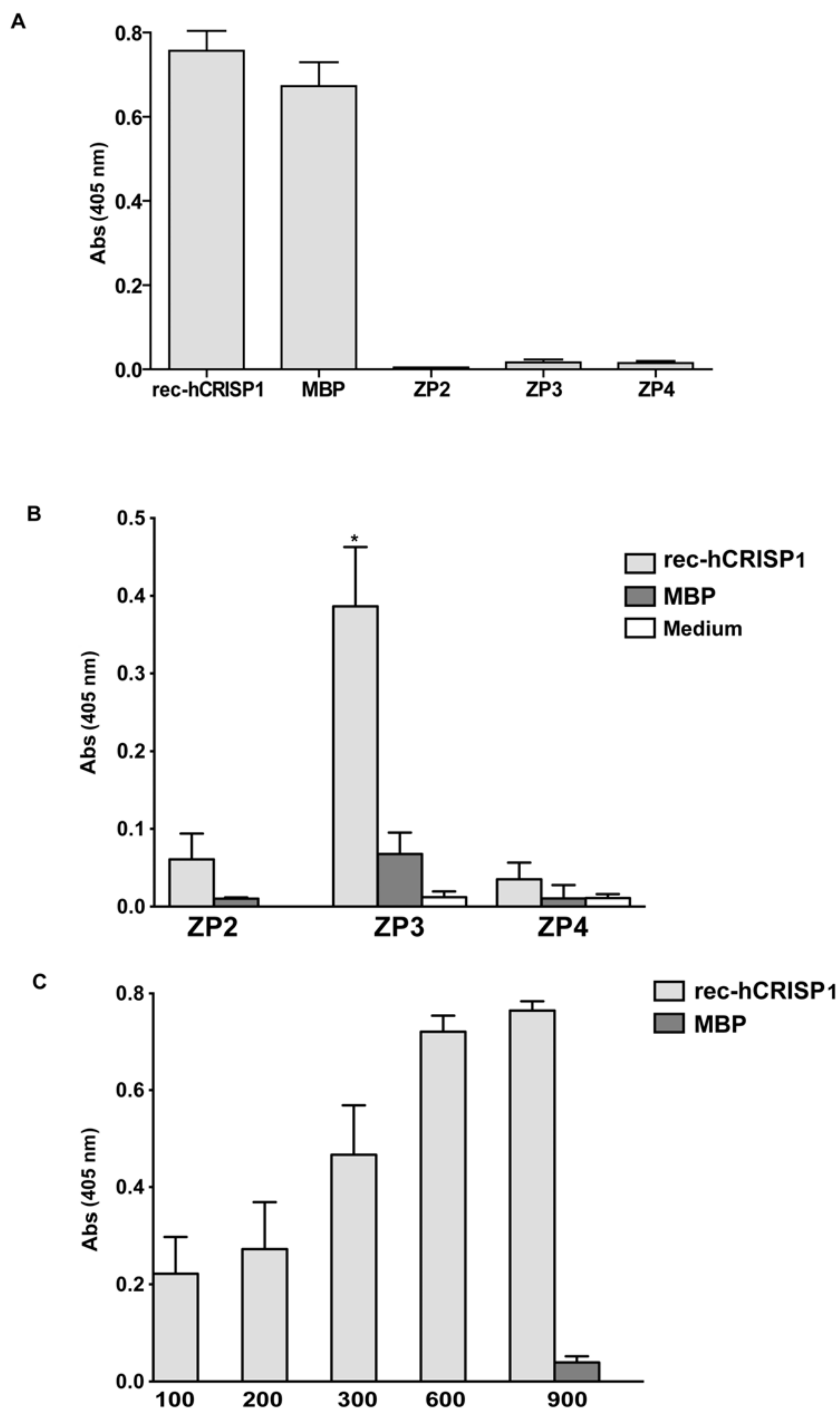

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