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Epididymal Protein CRISP1 Plays Different Roles During the Fertilization Process

Review

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ABSTRACT: Rat epididymal CRISP1, the first described member of the evolutionarily conserved Cysteine-RIch Secretory Protein (CRISP) family, is expressed in the proximal regions of the epididymis and associates with the sperm during epididymal transit. Evidence indicates the existence of 2 populations of CRISP1 in spermatozoa: a major one, loosely bound, which is released during capacitation and, therefore, proposed as a decapacitating factor; and a minor one, strongly associated with spermatozoa that remains on the cells after capacitation and is proposed to participate in gamete interaction. Originally localized to the dorsal region of capacitated sperm, CRISP1 migrates to the equatorial segment with capacitation and acrosome reaction. Consistent with these localizations, in vitro fertilization experiments support the involvement of CRISP1 in the first step of sperm–zona pellucida (ZP) interaction and subsequent gamete fusion through its interaction with egg-complementary sites. The potential roles of CRISP1 in capacitation and fertilization have been further supported by the finding that capacitated spermatozoa from CRISP1 "knockout" animals exhibit low levels of protein tyrosine phosphorylation and have an impaired ability to fertilize zona-intact and zona-free eggs in vitro. Moreover, recent evidence from mutant spermatozoa reveals that CRISP1 mediates the stage of sperm binding to the ZP. Altogether, these observations support the view that CRISP1 is a multifunctional protein playing different roles during fertilization through its different associations with and localizations on spermatozoa. We believe these results contribute to a better understanding of the molecular mechanisms involved in both the fertilization process and the acquisition of sperm-fertilizing ability that occurs during epididymal maturation.

Key words: Epididymis, sperm.
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In mammals, spermatozoa released from the testis are fully differentiated cells incapable of fertilizing an egg. To become competent to fertilize, mammalian spermatozoa must undergo several physiological changes during their transit through the male and female reproductive tracts, known as epididymal maturation and capacitation, respectively (Yanagimachi, 1994). Epididymal maturation is an androgen-dependent process during which spermatozoa undergo a series of biochemical and functional changes modulated by the epididymal luminal composition. Evidence supports the idea that epididymal maturation involves an extensive remodeling of the sperm plasma membrane, including the modification of pre-existing testicular molecules and the acquisition of new proteins of epididymal origin (Cuasnicú et al, 2002). Some of these proteins contribute to the stabilization of the sperm plasma membrane, which prevents the occurrence of premature capacitation, and are, therefore, known as "decapacitation factors," whereas others have been implicated in the acquisition of the sperm's ability to bind and recognize the egg. One of the more abundant proteins secreted by the rat epididymis is CRISP1, originally described by Cameo and Blaquier (1976) more than 30 years ago. This review will focus on the findings and recent advances made by our laboratory toward a better understanding of the functional role of this epididymal protein in the fertilization process.

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The CRISP Family

The major secretory protein of the rat epididymis was originally named DE because it ran as 2 clearly distinguishable bands in nondenaturing gels, proteins D and E (Cameo and Blaquier, 1976). These 2 proteins are the product of the same gene (Klemme et al, 1999); cannot be separated on the basis of their solubility, molecular weight, or charge (Garberi et al, 1979; Brooks, 1982); and are considered as a single entity.

DE is now known as CRISP1 for being the first-described member of the CRISP (Cysteine-RIch Secretory Protein) family, a large group of secreted proteins with molecular weights of about 20–30 kDa and characterized by the presence of 16 conserved cysteine residues, 10 of which are clustered in the C-terminal region of the molecule. In recent years, this family has grown because of the discovery of new related proteins found not only in mammals but also in phylogenetically more distant organisms. Besides CRISP1, other members of the family have been identified in the mammalian male reproductive tract: CRISP2, also known as Tpx-1, which is expressed in the testis and is synthesized in developing spermatids (Hardy et al, 1988; Kasahara et al, 1989); CRISP3, with a wider tissue distribution, including reproductive (ie, prostate and ovary) and nonreproductive (ie, salivary gland, pancreas, thymus, and colon) organs (Haendler et al, 1993; Kjeldsen et al, 1996; Udby et al, 2005); and CRISP4, exclusively expressed in the epididymis (Jalkanen et al, 2005). CRISP members are also present in salivary secretions of certain snakes, lizards, and snails (Morrissette et al, 1995; Milne et al, 2003; Yamazaki and Morita, 2004) and in *Xenopus* and chicken embryos (Olson et al, 2001; Smith et al, 2001; Schambony et al, 2003). The crystallographic analysis of several CRISP family members revealed them to be modular proteins formed by 2 domains—a plant pathogenesis-related domain (PR-1) and a cysteine-rich domain (CRD)—connected by a short hinge (see Cohen et al, 2008; Gibbs et al, 2008).

More distantly related proteins with significant homology with the *N*-terminal PR-1 domain of CRISPs, but lacking or presenting truncated forms of the CRD, have also been described. Within this group are found Ag5, a family of very abundant and allergenic proteins present in vespid venoms, and PR-1 (pathogenesis-related 1) plant proteins mainly expressed in response to pathogen invasion (Hendriksen et al, 1996; Fernandez et al, 1997). More recently, these types of truncated forms of CRISP-related proteins have also been described in mammals and include the GLIPR1 (glioma PR-1) and the GAPR1 (Golgi-associated PR-1) families. All these proteins are grouped together into the CAP (CRISP/Antigen5/PR-1) superfamily (see Gibbs et al, 2008).

Association of CRISP1 With Spermatozoa During Epididymal Maturation

Immunodetection techniques with a polyclonal antibody against CRISP1 have indicated that this protein is synthesized in the proximal segments of the rat epididymis and associates with the sperm surface during epididymal maturation, localizing to the dorsal region of the sperm head (Kohane et al, 1980a,b).

In an attempt to gain insights into the interaction of CRISP1 with the sperm surface, rat cauda epididymidal sperm were subjected to different treatments known to remove peripheral and integral proteins. Results indicated that CRISP1 associates with spermatozoa with 2 different affinities: most CRISP1 is loosely associated with the sperm surface and is removed by ionic strength, whereas a minor fraction of the protein is strongly bound to the cell and behaves as an integral membrane protein (Cohen et al, 2000). Quantification of CRISP1 in caput, corpus, and cauda spermatozoa before and after exposure to high concentrations of NaCl revealed that although the amount of CRISP1 resistant to the treatment was similar in the 3 regions, the protein released by high ionic strength was significantly greater in spermatozoa from the successive regions, suggesting that CRISP1 associates strongly with the cells in the proximal regions of the organ and then loosely during epididymal transit.

To establish the mechanisms involved in the association of CRISP1 with spermatozoa, in vitro binding experiments examined the loading of caput or cauda cells with purified or recombinant CRISP1 under different experimental conditions. The lack of binding of CRISP1 to spermatozoa observed in all the cases led us to examine whether the CRISP1-sperm interaction requires the participation of other epididymal components. In this regard, recent experiments performed in our laboratory indicate that zinc, present in high concentrations in the rat epididymis (Mawson and Fischer, 1951; Stoltenberg et al, 1996), is involved in the weak association of CRISP1 with rat spermatozoa (unpublished data). In parallel to these observations, recent evidence revealed the presence of CRISP1 in small membrane vesicles known as "epididymosomes" within epididymal fluid. Protein extraction showed that CRISP1 on these vesicles behaves as the population tightly bound to sperm (Maldera, unpublished), opening the possibility that epididymosomes are involved in the mechanism by which the strongly bound CRISP1 population associates with spermatozoa.

Behavior of CRISP1 During Capacitation

Capacitation experiments of rat spermatozoa under in vivo or in vitro conditions have shown that a substantial amount of CRISP1 is released from spermatozoa during this process, suggesting that the protein acts as a decapacitation factor (Kohane et al, 1980b). Subsequent experiments have demonstrated that part of the protein remains on spermatozoa after capacitation (Cameo et al, 1986), migrates to the equatorial segment with the acrosome reaction (Rochwerger and Cuasnicu, 1992),

and participates in gamete interaction (Rochwerger et al, 1992; Busso et al, 2007a).

Several observations support the conclusion that CRISP1 released during capacitation corresponds to the loosely bound population removable by ionic strength, whereas the protein remaining on spermatozoa after capacitation and migrating to the equatorial segment corresponds to the tightly bound population. First, the amount of CRISP1 released from mature spermatozoa during capacitation is not significantly different from that released by physiological saline treatment. Second, no further protein can be extracted by high ionic strength once spermatozoa are capacitated, indicating that the loosely bound protein had been released during capacitation. Finally, the majority of capacitated spermatozoa lacking the loosely bound CRISP1 population has the protein in the equatorial segment (Cohen et al, 2000).

In addition to our findings describing the release of CRISP1 during capacitation, evidence from another group has shown an inhibitory effect of CRISP1 on rat sperm protein tyrosine phosphorylation and the progesterone-induced acrosome reaction when it is present during capacitation, further supporting the idea that CRISP1 plays a role as a decapacitating factor (Roberts et al, 2003).

As another approach to elucidate the functional role of CRISP1 in fertilization, a mouse line containing a targeted disruption of the *Crisp1* gene was generated. *Crisp1*$^{-/-}$ mice were found to be fertile (Da Ros et al, 2008), a result that adds CRISP1 to a growing list of proteins proposed to be involved in fertilization but showing only mild or no fertility defects when knocked out (Okabe and Cummins, 2007). Because many of these fertile knockout mice often exhibit other reproductive deficiencies (Lu and Shur, 1997; Baba et al, 2002), *Crisp1*$^{-/-}$ males were evaluated for evidence of changes in different functional sperm parameters. In view of the proposed decapacitating activity of CRISP1, we first investigated whether the absence of the protein in the mutant mice produced an increase in the levels of tyrosine phosphorylation and acrosome reaction, 2 capacitation-dependent events. Surprisingly, capacitated spermatozoa from *Crisp1*$^{-/-}$ mice not only exhibited normal levels of spontaneous and progesterone-induced acrosome reactions but presented clearly lower levels of tyrosine phosphorylation than controls (Da Ros et al, 2008). This represented the first report showing a significant decrease in sperm protein tyrosine phosphorylation accompanied by normal levels of fertility, suggesting that protein tyrosine phosphorylation is either not required or required in low levels to achieve acrosome reaction and normal fertility.

Our observations in knockout mice raised the possibility that CRISP1 plays a regulatory role during mouse sperm capacitation different from that previously proposed for the rat. In this regard, we have recently observed that the presence of CRISP1 during rat sperm capacitation produced an inhibition on the spontaneous acrosome reaction similar to that observed when spermatozoa were capacitated in the presence of cauda epididymidal plasma (CEP), a fluid with known decapacitating activity (Figure 1). Additionally, spermatozoa incubated with CRISP1 during capacitation were unable to fuse with zona-free eggs (control: 60% ± 2%, CRISP1 6 μM: 0%, CEP 300 μg of protein/mL 0%; $P < .001$ vs control). Together, these observations support a role for CRISP1 during rat sperm capacitation. From the finding that the CRD of CRISP proteins from snake venoms, as well as testicular protein CRISP2, are able to regulate different types of ion channels (Guo et al, 2005; Gibbs et al, 2006), it is possible that rat CRISP1 acts as a decapacitation factor through an ion channel regulatory activity located in the CRD of the molecule.

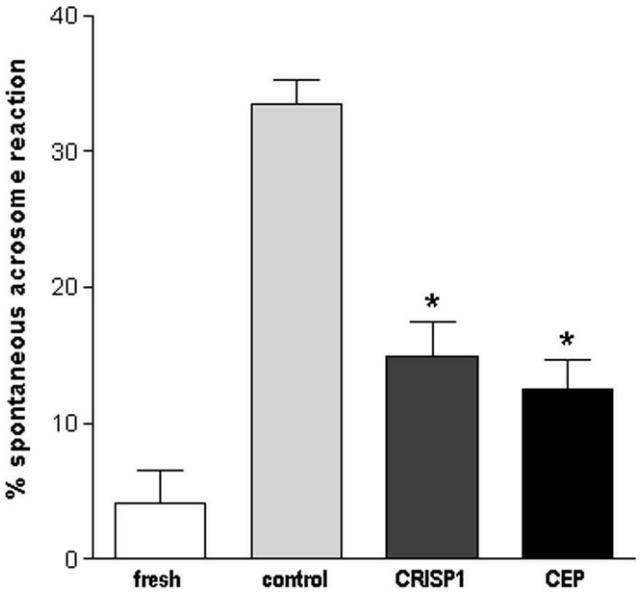

Figure 1. Effect of CRISP1 on the spontaneous acrosome reaction. Fresh rat cauda epididymidal spermatozoa or spermatozoa incubated for 5 hours under capacitating conditions in the absence (control) or presence of 6 μM CRISP1 or 300 μg of protein/mL of cauda epididymidal fluid (CEP), were fixed and stained with Coomassie brilliant blue to evaluate the percentage of acrosome-reacted cells. Results are the mean ± SEM of 4 independent experiments. * $P <$ .0001 vs control.

Participation of CRISP1 in Sperm-Egg Interaction

The relocation of CRISP1 to the equatorial segment, the region through which acrosome-reacted sperm fuse with the egg plasma membrane (Bedford et al, 1979; Yanagimachi, 1994), led us to study a possible role of CRISP1 in gamete fusion. Exposure of zona-free rat

eggs to purified CRISP1 before insemination with capacitated spermatozoa significantly reduced the percentage of penetrated eggs, with no effects on sperm-egg binding, supporting the participation of this protein in an event subsequent to binding and leading to fusion (Rochwerger et al, 1992). This possibility was subsequently confirmed by the use of the Hoechst dye transfer technique, which specifically evaluates the sperm-egg fusion event (Cohen et al, 1996). The inhibitory effect of CRISP1 on gamete fusion also revealed the existence of complementary sites for CRISP1 on the oocyte, which were localized over its entire surface with the exception of the area overlying the meiotic spindle, a region at which fusion rarely occurs (Rochwerger et al, 1992; Cohen et al, 1996). Thus, while CRISP1 is localized in the fusogenic region of sperm, the corresponding binding sites are localized in the fusogenic region of the oocyte.

Structure-function studies in which a recombinant CRISP1 protein expressed in bacteria (recCRISP1) was added during rat gamete co-incubation showed that the egg-binding activity of the protein does not involve carbohydrates and resides in the polypeptide region of the molecule (Ellerman et al, 2002). The use of a series of recombinant fragments of CRISP1 circumscribed this activity to a region of 45 amino acids (114–158) that, interestingly, contain the 2 feature motifs of the CRISP family: Signature 1 (S1) and Signature 2 (S2) (Ellerman et al, 2006). Localization and gamete fusion experiments with 2 synthetic peptides, with the amino acid sequences corresponding to each of the motifs, showed that only the peptide corresponding to S2 was capable of binding to the egg and interfering with gamete fusion. The lack of inhibition observed with a scrambled peptide confirmed the relevance of the S2 region for the binding of CRISP1 to the egg (Ellerman et al, 2006). These findings indicate that the egg-binding ability of CRISP1 resides within the PR-1 domain of the molecule, supporting the idea that the different biological roles proposed for CRISP1 reside in different domains of the protein.

The postulated involvement of CRISP1 in gamete fusion was further supported by in vitro assays showing a significant reduction in the fusion ability of *Crisp1*$^{-/-}$ spermatozoa compared with heterozygous spermatozoa (Da Ros et al, 2008). Moreover, when zona pellucida (ZP)-free eggs were simultaneously inseminated with *Crisp1*$^{+/+}$ and *Crisp1*$^{-/-}$ spermatozoa in a competition assay, the mutant spermatozoa exhibited an even lower fusion ability (Da Ros et al, 2008), confirming that spermatozoa lacking CRISP1 have a clear disadvantage in their ability to fuse with the egg. Our results also showed that the fusion ability of *Crisp1*$^{-/-}$ spermatozoa could be further inhibited by the presence of CRISP1 or CRISP2 during gamete co-incubation (Da Ros et al, 2008), suggesting that another CRISP present in sperm cooperates with CRISP1 during fertilization and compensates for the lack of CRISP1 in the mutant mice. In this regard, previous results of our laboratory, indicating that CRISP2 is involved in sperm-egg fusion in mice and humans (Busso et al, 2005, 2007b), support the existence of a functional cooperation between these homologous proteins and the view that CRISP2 is a candidate molecule that compensates for the lack of CRISP1 in the knockout mice to ensure the success of fertilization.

Although the findings described earlier strengthen the idea of the participation of CRISP1 in gamete fusion, circumstantial evidence has suggested that this protein plays an additional role during fertilization. In vivo observations have shown that rat spermatozoa exposed to a serum against CRISP1 before uterine insemination exhibit a significantly lower fertilizing ability than untreated spermatozoa (Cuasnicu et al, 1984; Perez Martinez et al, 1995). However, unlike what would be expected for a protein involved only in gamete fusion, the nonfertilized eggs did not accumulate spermatozoa in the perivitelline space, indicating that the antibodies interfered with an event previous to sperm-egg fusion. Subsequent in vitro fertilization experiments with zona-intact rat and mouse eggs revealed that the presence of either anti-CRISP1 polyclonal antibody or native CRISP1 during gamete co-incubation significantly decreased the percentage of fertilized eggs with no accumulation of perivitelline spermatozoa, supporting the view that this inhibition occurred at the sperm-ZP interaction (Busso et al, 2007a). The specific evaluation of the effect of anti-CRISP1 antibodies or CRISP1 protein on the number of spermatozoa bound per egg indicated that the protein is involved in the initial step of sperm binding to the ZP (Busso et al, 2007a). The presence of recombinant, instead of native, CRISP1 during gamete co-incubation also inhibited egg fertilization although, in this case, with an increase in the number of perivitelline spermatozoa (Busso et al, 2007a). In agreement with this, indirect immunofluorescence experiments revealed that native CRISP1 was capable of binding to both the ZP and the egg plasma membrane, whereas recCRISP1 could bind only to the egg surface. The failure of recCRISP1 to interact with the ZP suggested that carbohydrates or the protein conformation are important for the association of CRISP1 with the ZP. In this regard, the finding that deglycosylated CRISP1 behaves as the untreated protein, whereas the heat-denatured protein associates only with the egg plasma membrane, favors the relevance of the CRISP1 protein conformation for sperm-ZP binding (Busso et al, 2007a).

The involvement of CRISP1 in the sperm-ZP interaction is further supported by in vitro fertilization

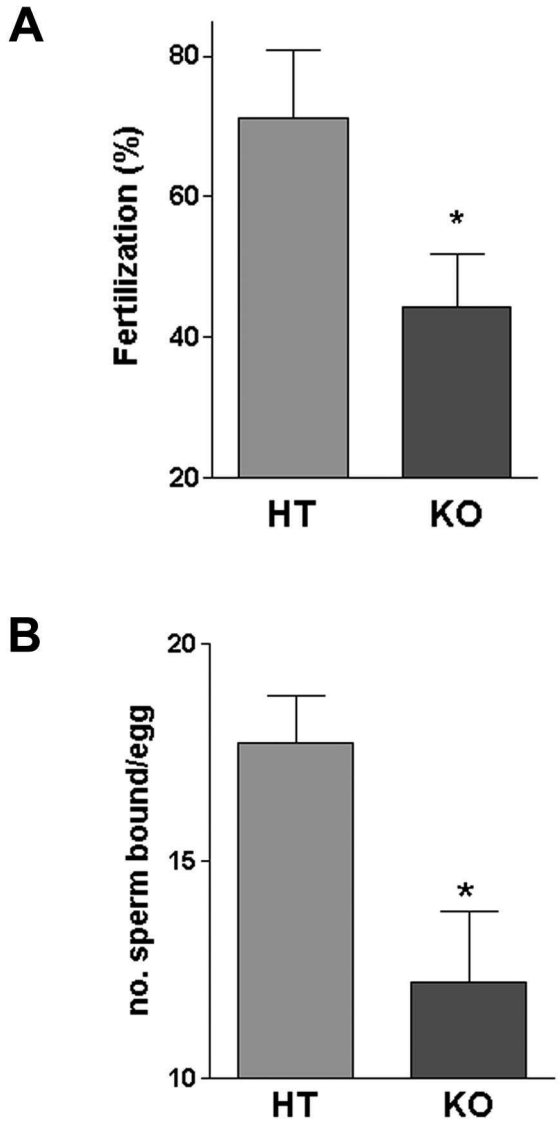

Figure 2. Evaluation of the ability of *Crisp1*$^{-/-}$ spermatozoa to interact with the zona pellucida. Zona-intact eggs were inseminated with *Crisp1*$^{+/-}$ (heterozygous [HT]) and *Crisp1*$^{-/-}$ (knockout, KO) capacitated spermatozoa. **(A)** After 3 hours of co-incubation, eggs were stained with Hoechst 33342 and evaluated for evidence of sperm penetration. Results are the mean ± SEM of 6 independent experiments. * $P = .0002$ vs HT. **(B)** After 30 minutes of co-incubation, eggs were recovered and washed, and the number of spermatozoa bound per egg was determined. Results are the mean ± SEM of 3 independent experiments. * $P < .05$ vs HT.

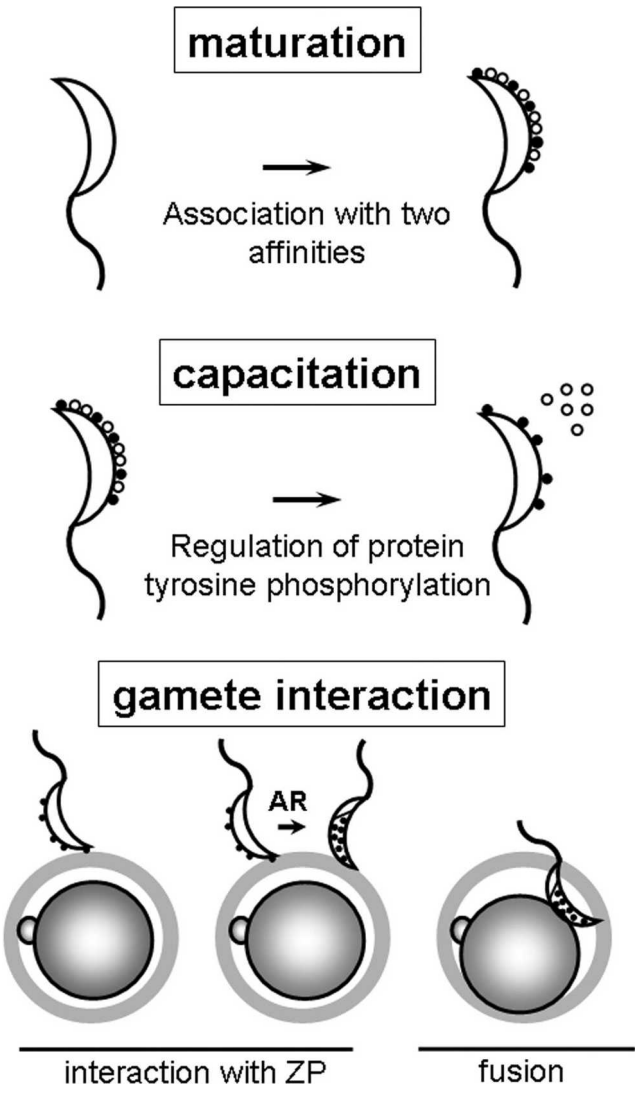

Figure 3. Schematic representation of the roles of CRISP1 in different steps of fertilization. During epididymal maturation, CRISP1 associates with the sperm surface with 2 different affinities: (○) loosely bound population; (●) tightly bound population. During capacitation, the loosely bound population is released from spermatozoa where it acts as a decapacitating factor by regulating protein tyrosine phosphorylation. The tightly bound population remains on spermatozoa after capacitation and is involved in different stages of the gamete interaction process: when localized in the dorsal region of the sperm head, it participates in sperm–zona pellucida (ZP) binding through a protein conformation–mediated mechanism. Then the ZP induces the acrosome reaction (AR) and the migration of CRISP1 to the equatorial segment, where it participates in sperm-egg fusion through the Signature 2 region of the molecule.

assays showing that *Crisp1*$^{-/-}$ spermatozoa exhibit lower penetration of ZP-intact eggs than wild-type spermatozoa with no accumulation of spermatozoa in the perivitelline space. Moreover, recent results have shown that the number of mutant spermatozoa bound per zona was significantly lower than controls (Figure 2), consistent with the reported role for CRISP1 in the initial step of sperm-ZP binding.

Conclusions

The results described led us to propose that CRISP1 is a multifunctional sperm protein playing different roles during the fertilization process. CRISP1 associates with the sperm surface with 2 different affinities during epididymal maturation (Figure 3). The weakly bound

population is released during capacitation and is very likely involved in this process by regulating protein tyrosine phosphorylation. The strongly bound population of CRISP1 remains localized on the dorsal region of capacitated intact spermatozoa and participates in the first step of sperm binding to the ZP through a conformation-mediated mechanism. Then the ZP induces both the sperm acrosome reaction and the migration of CRISP1 to the equatorial segment, where it mediates gamete fusion through the interaction of its S2 motif with complementary sites on the egg surface. These observations, together with our results showing that testicular CRISP2 is involved in sperm-egg fusion (Busso et al, 2005; Busso et al, 2007b), support the idea of different functional roles for the same CRISP and the involvement of different CRISPs in the same fertilization event. Because spermatozoa are transcriptionally inactive cells, this protein functional redundancy likely has evolved as a mechanism to ensure the success of fertilization.

We believe these results contribute to a better understanding of the molecular mechanisms involved in both the fertilization process and the acquisition of sperm fertilizing ability during epididymal maturation.

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